National Library of Canada
Canadian Theses Service
Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Service des thèses canadiennes

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canada

UNIVERSITY OF ALBERTA

EFFECTS OF HEMOPOIETIC GROWTH FACTORS ON THE GROWTH, DIFFERENTIATION AND FUNCTION OF NORMAL AND TRANSFORMED HUMAN TROPHOBLASTS.

BY

MARIA INES GARCIA-LLORET

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

IN

EXPERIMENTAL MEDICINE
DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA
SPRING, 1991

National Library
of Canada

Canadian Theses Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale
du Canada

Service des thèses canadiennes

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-66601-3

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: MARIA INES GARCIA-LLORET

TITLE OF THESIS: EFFECTS OF HEMOPOIETIC GROWTH FACTORS ON THE GROWTH, DIFFERENTIATION AND FUNCTION OF NORMAL AND TRANSFORMED HUMAN TROPHOBLASTS.

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1991

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts may be printed or otherwise reproduced without the author's written permission.

(signed) ______________________

Permanent Address:
Edificio 18, No 302
Villa Olimpica
Tlalpan c.p. 14020
Mexico, D.F., Mexico

Date: April 19, 1991

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Effects of Hemopoietic Growth Factors on the Growth, Differentiation and Function of Normal and Transformed Human Trophoblasts** submitted by **Maria Ines Garcia-Lloret** in partial fulfillment of the requirements for the degree of **Master of Science** in Medical Sciences (Medicine).

A.R. Turner

L.J. Guilbert

D.W. Morrish

A. Janowska-Wieczorek

A.R.E. Shaw

Date: April 19, 1991

ABSTRACT

The placenta is a complex multifunctional organ interposed between the maternal and fetal tissues. During the formation of the placenta, the trophoblast, its most specialized tissue, proliferates, differentiates, invades maternal structures and secretes a variety of substances thought to be essential for the initiation and maintenance of pregnancy. The molecular mechanisms that regulate the development and function of the human trophoblast are poorly understood, but there is increasing evidence from animal studies that hemopoietic colony-stimulating factors may play an important role in these processes.

In this thesis, I have studied the effects of the hemopoietic colony-stimulating factors (CSFs), macrophage CSF (CSF-1), and granulocyte-macrophage CSF (GM-CSF) on the proliferation and differentiation of normal and malignant human trophoblasts *in vitro*. I present experimental evidence showing that both normal and malignant trophoblasts express functional CSF-1 receptors and are able to respond to this factor, the former by morphological differentiation and increased peptide hormone secretion and the latter by proliferation. GM-CSF was also a potent differentiation inducer for normal trophoblasts, whereas no effect could be demonstrated on malignant cells. Three well characterized choriocarcinoma cell lines were used as a model of the fully transformed state. All lines secreted CSF-1 and GM-CSF in culture. Anti GM-CSF anti CSF-1 receptor antibody inhibited their proliferation *in vitro*, suggesting that CSFs mediated autocrine mechanisms might be involved in the malignant transformation of the human trophoblast.

Taken together, the data presented in this thesis indicate that the hemopoietic colony-stimulating factors CSF-1 and GM-CSF are trophoblast targeting cytokines, demonstrating novel functions for these versatile cytokines outside the hemopoietic system.

ACKNOWLEDGEMENTS

To my husband and children, for their unconditional support.

To Dr. Larry Guilbert, for everything, from intellectually motivating scientific discussions to counseling in moments of experimental frustration, and above all for his infinite patience with this less than ideal graduate student.

To Dr. A.R. Turne,r for his many helpful comments that greatly collaborated to the final completion of this thesis.

To Dr. D.W. Morrish , an enthusiastic collaborator and a conscientious scientist, for performing the many peptide hormone assays .

To Bonnie Lowen and Anne Smith, for their expert technical assistance and their friendship.

To Debbie Neufeld, for her excellent secretarial work and cheerful mood, even under the highest pressure.

To the Canadian Red Cross Society, for their financial support.

To Professor E.B. Dowdle, for initiating me in the marvelous road of scientific research.

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION 1

1.1 Placental Development 2

1.2 Placental Function 10

1.3 Hemopoietic Growth Factors and the Placenta 19

1.4 Placental Pathology 28

1.5 Conclusions 55

1.6 Research Proposal & Experimental Approach 37

Bibliography 39

CHAPTER 2 FIRST SEPARATE STUDY: HEMOPOIETIC COLONY STIMULATING FACTORS INDUCE THE DIFFERENTIATION OF NORMAL HUMAN TROPHOBLASTS. 55

Bibliography 72

CHAPTER 3 SECOND SEPARATE STUDY: CHORIOCARCINOMA CELLS ARE AUTOCRINE FOR TWO HEMOPOIETIC COLONY-STIMULATING FACTORS. 74

Bibliography 101

CHAPTER 4 GENERAL DISCUSSION & CONCLUSIONS 105

Bibliography 121

LIST OF TABLES

Table 2.1	Binding of ^{125}I-CSF-1 to normal trophoblasts as function of time at 4° C.	71
Table 3.1	Comparison of unoccupied ^{125}I-CSF-1 binding sites on choriocarcinoma cell lines under saturation binding conditions, 4° C.	94
Table 3.2	Effect of growth factors on the clonogenic growth of human choriocarcinoma cell lines.	95
Table 3.3	CSF-1 concentrations in choriocarcinoma cell preparations.	96
Table 3.4	Inhibition of choriocarcinoma proliferation by anti CSF-1 receptor antibodies.	97
Table 3.5	GM-CSF production by choriocarcinoma cell lines.	98
Table 3.6	Detection of GM-CSF by CML growth.	99
Table 3.7	Inhibition of choriocarcinoma cell line growth by anti GM-CSF antibody.	100

LIST OF FIGURES

Figure 2.1 Localization of c-*fms* mRNA in first trimester human placental villi by *in situ* hybridization. 62

Figure 2.2 Expression of high affinity binding for ^{125}I-CSF-1 on cultured human term trophoblasts. 64

Figure 2.3 Effect of CSF-1 and GM-CSF on trophoblast morphological differentiation.

- a - Control 66
- b - CSF-1 67
- c - GM-CSF 68

Figure 2.4 Effect of CSF-1 and GM-CSF on hCG and hPL production by human term trophoblasts. 70

Figure 3.1 Expression of CSF-1 receptor mRNA in normal trophoblasts and choriocarcinoma cell lines. 93

LIST OF ABBREVIATIONS

5/10.14 - CSF-1 dependent macrophage cell line
ACTH - Adrenocorticotrophin
BeWo - Choriocarcinoma cell line
C - Celsius
CC - Choriocarcinoma
c-*fms* - Cellular oncogene encoding the receptor to CSF-1
CHO cells - Chinese hamster ovary cells
CML - Chronic myelogenous leukemia
CO - Carbon dioxide
cpm - counts per minute
CSA - Colony-stimulating activity
CSFs - Colony-stimulating factors
CSF-1 - Colony-stimulating factor-1
CT - Cytotrophoblast
EGF - Epidermal growth factor
FBS - Fetal bovine serum
FCS - Fetal calf serum
FSH - Follicle-stimulating hormone
GF - Growth factors
GM-CSF - Granulocyte-macrophage colony-stimulating factor
GTD - Gestational trophoblastic disease
G-CSF - Granulocyte colony-stimulating factor
HCG - Human chorionic gonadotrophin

HCSFs	Hemopoietic colony-stimulating factors
HGF	Hemopoietic growth factor
HM	Hydatidiform mole
HPCM	Human placental cell conditioned medium
HPL	Human placental lactogen
hrs	Hours
^{3}HTdr	Tritiated thymidine
^{125}I-CSF-1	Iodine 125 labeled CSF-1
ICM	Inner cell mass
IGF	Insulin growth factor
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-6	Interleukin-6
IMB	IMDM lacking bicarbonate adjusted to pH 7.35 with
IMDM	Iscove's modified Dulbecco's medium
IU	International units
JAR	Choriocarcinoma cell line
JEG-3	Choriocarcinoma cell line
Kb	Kilobase
Kd	Dissociation equilibrium constant
L	Litre
L cell	Murine fibroblast cell line
LH	Luteinizing hormone
LIF	Leukemic inhibitory factor

LPS - Lipopolysaccharide

M - Mole

MGFs - Macrophage growth factors

mL - Milliliter

mm - millimeter

mM - millimolar

M-CSF - Macrophage colony-stimulating factor

NaCL - Sodium chloride

NaOH - Sodium hydroxide

nM - nanomolar

MEM - Minimum essential medium

P388D1 - CSF-1 receptor-bearing murine macophage cell line

PBS - Phosphate buffered saline

PDGF - Platelet derived growth factor

rpm - Revolutions per minute

RRA - Radioreceptor assay

SP-1 - Pregnancy-specific protein 1

ST - Syncytiotrophoblast

STH - Sommatotrophin

TNF - Tumor necrosis factor

TSH - Thyroid-stimulating hormone

μCi - Microcurie

μg - Microgram

μl - Microliter

u/ml - units per milliliter

uPA	-	urokinase-type plasminogen activator
v/v	-	volume per volume

CHAPTER 1 INTRODUCTION

1.1 PLACENTAL DEVELOPMENT

Both the placenta and the fetus arise from the same single cell, the fertilized ovum. Within hours of fertilization, the zygote begins to divide (Beaconsfield *et al.*, 1980). The mechanisms that initiate this first cell division are poorly understood, but the fact that both fertilization and early embryo development have been achieved *in vitro* suggests that no specific exogenous signals are essential for the normal occurrence of these phenomena (Sherman, 1978). It has been proposed that given a suitable environment (an ill defined progestational fluid secreted by the endometrium in response to progesterone), the embryonic cells divide purely as a result of fertilization and zygote formation. DNA synthesis is accompanied by active translation of mRNA transcripts: by the eight cell stage more than a thousand kinds of proteins are present in the embryo (Aitken *et al.*, 1979).

The cluster of dividing cells moves down oviduct reaching the uterine cavity about four days after fertilization. At this point changes have already begun that will culminate with the formation of the blastocyst. The blastocyst consists of a single layer of cells, the trophoblast, surrounding a fluid filled cavity, the blastocoele, which contains a mass of cells at one end, the inner cell mass (ICM) (Boyd & Hamilton, 1974). The ICM is the forerunner of the fetus and the trophoblast subsequently contributes to the formation of the placenta. Once in the uterine cavity, the trophoblast comes in intimate association with the maternal endometrium and the blastocyst proceeds to implant in the uterine wall around seven days after fertilization.

The implantation of the blastocyst in the wall of the uterus is a remarkable and poorly understood process, at the end of which the blastocyst becomes completely buried in the endometrial tissue (Pijneborg *et al.*, 1981). The mechanisms that initiate, regulate and limit the position, adhesion and invasion of maternal tissues by the outer layer of the blastocyst (the trophoblast) remain largely unknown. However, experimental evidence suggests that both maternal and embryonic factors can influence the normal development of these processes. If pregnant rats are ovariectomized shortly after mating no implantation occurs. This effect can be reversed by the combined administration of estrogen (E) and progesterone(P) (Mc Cormack & Glasser, 1983). Progesterone seems to maintain the viability of the embryo whereas estrogen has been shown to increase the permeability of, and induce the secretion of prostaglandins by, endometrial tissue (Inskeep *et al.*, 1983). While both hormones have been implicated in the cyclic changes of the endometrium that take place during the female menstrual cycle, the requirements for E and P for implantation vary in different species. Moreover, since implantation can occur outside of the uterus, the presence of a hormonally primed permissive endometrium appears not to be an absolute requirement.

The ability to invade neighboring tissues is a property conspicuously shared by normal trophoblasts and cancer cells (Yagel *et al.*, 1988). Several mechanisms have been proposed to explain the physiological basis of invasion. Increased adhesion to substrates, decrease mutual adhesion, inhibition of growth of normal cells in the region of the invasion and liberation of lytic substances, are all well documented characteristics of neoplastic cells. While plasminogen activators have been shown to be produced during early mouse development suggesting, the involvement of these proteases in the invasion of the endometrium (Strickland & Reich, 1976), none of these properties has been clearly demonstrated for normal trophoblasts. In contrast to tumor cells, invasion by trophoblasts is a self limited, finely regulated process. Whether maternal factors are able to modulate its initiation and limit its progression or whether it is a totally autonomous function of the trophoblast is totally unknown. Interestingly, implantation occurs faster and deeper in ectopic sites, suggesting some limitations imposed by the endometrial host tissue (Psychoyos, 1974).

It is during the early stages of implantation that the invasive trophoblast begins to differentiate into two cell layers, the outer syncytiotrophoblast (ST) and the inner cytotrophoblast (CT) (Aitken *et al.*, 1979). As the ST advances into the endometrium, it becomes vacuolated creating a system of small cavities in which maternal blood from the uterine arteries eventually flows (Robertson, 1976). The degeneration of endometrial cells that achieves the opening of these blood vessels is presumably facilitated by proteases secreted by the advancing trophoblast. Human embryonic tissue comes in direct contact with maternal blood when the trophoblast actually invades the spiral arterioles that lie in the uterine wall (Robertson *et al.*, 1974). This intimate contact defines hemochorial placentas (Ramsey, 1975). Complete development of the placenta is achieved by the simultaneous occurrence of two processes, the proliferation and differentiation of trophoblast and of chorionic villi structures and the response of the uterine lining that gives rise to the decidua (Nehemiah *et al.*, 1981).

The Decidua

The human endometrium is a dynamic mucosa that undergoes a cyclic process of regression and renewal even in the absence of fertilization, as evidenced by the periodic changes in the cellular and extracellular matrix components observed in the stromal compartment of the uterus (Glasser & Mc Cormack, 1980). While some of these changes appear to be under the direct control of the ovarian hormones estrogen and progesterone which in turn are regulated by the coordinated action of hypophyseal peptides (Martin & Finn, 1968), others like the transient migration of leukocytes into the uterine stroma (Bulmer & Sutherland, 1983), cannot be fully explained by this mechanism.

As mentioned before, implantation and subsequent development of the human placenta depend on certain changes in the endometrium that culminate in the formation of the decidua. The human trophoblast, which is the most invasive known, elicits the most pronounced decidual response and as a result, the closest possible link is established between the mother and the fetus (Wynn, 1971). Decidualization occurs gradually and is not completed until several days after nidation. It can also be induced in hormonally primed pseudopregnant uterine endometrium by mechanical and chemical stimuli (Pollard *et al*, 1987). During the development of the decidual reaction, the endometrial stromal components enlarge to form distinctive polygonal cells with large vesicular nuclei and translucent membranes. The decidua directly beneath the implantation site is called decidua basalis (Panigel, 1986). Surrounding the ovum and separating it from the rest of the uterine cavity is the decidua capsularis. The remainder of the uterus is lined by the denominated decidua parietalis. Extensively invaded by the trophoblast, it is the decidua basalis that enters into the formation of the placenta itself.

Several biological functions have been attributed to decidual cells: a nutritive role for the embryo, secretion of prolactin, maintenance of pregnancy by protecting maternal tissues from destructive invasion, secretion of immunoregulatory substances to prevent allograft rejection, and production of hormones that actively promote the growth and differentiation of the trophoblast (Kearns & Lala, 1982). However, confirmatory evidence for most of these functions has not been presented. Moreover, it is not clear whether decidual reaction is necessary at all for the maintenance of pregnancy, since normal fetuses can fully develop outside of the uterus.

The Trophoblast

The trophoblast is a specialized tissue unique to mammals that has developed in response to the needs of viviparity. While its name implies a nutritive function, there is increasing evidence that the trophoblast is involved in the wide variety of metabolic, endocrine and immunological mechanisms that contribute to initiation and maintenance of a normal pregnancy.

Up to the eight cell stage the individual blastomeres are distinct and undergo regular cleavage appearing to be similar in gross morphology. Thereafter, the cell outlines become less clear, desmosomes differentiate between adjacent outer cells later forming junctions. These junctional changes in the outer cell layer may be the first indication of the formation of the trophoblast (Aitken *et al.*, 1979). The mechanisms that induce this early differentiation of blastomeres into the different structures that compose the blastocyst (inner cell mass and trophoblast) are not known. It has been proposed that this is an autonomous process, not requiring maternal stimuli and that differentiation starts when there are sufficient blastomeres (8 to 16) to form two cell layers so that the outer cells form the trophoblast and the inner the ICM as evidenced by aggregation chimeras studies. This suggests that component blastomeres differentiate according to their position (Gardner & Rossant, 1976).

Normal trophoblast develops from the outer cell layer of the blastocyst and by the seventh day has usually differentiated into two layers, an inner layer of mononuclear cytotrophoblasts and an outer layer of multinucleated syncytiotrophoblast. The ST is formed by fusion of CT cells and is in itself incapable of cell division. It is a real syncytium as confirmed by electron microscopy (Kaufmann & King, 1982). Other specific features are the presence of surface microvilli and of a well developed endoplasmic reticulum typical of actively secreting tissues. Indeed, the ST has been shown to be the main site of synthesis of placental products such as human chorionic gonadotrophin (HCG), human placental lactogen (HPL) and steroid hormones. In contrast, typical CT are ultrastructurally simple, with large nuclei and prominent nucleoli, large mitochondrias and few Golgi bodies. These characteristics are usually present in embryonic and neoplastic cells, the principal function of which is growth and proliferation rather than elaboration of specialized endocrine or exocrine products.

As mentioned before, further proliferation of the syncytio and cytotrophoblast occurs early during its invasion of the endometrium. Whether this initial proliferation and differentiation of the trophoblast is controlled by maternal factors is controversial. Species vary considerably in the time interval between the formation of the blastocyst and its implantation and also in the extent of growth during this period (Aitken *et al.*, 1979). The human embryo implants relatively early, between the 6th and the 7th day after fertilization and the blastocyst grows little after the 5th day, suggesting that growth stimulatory factors present within the maternal tissue are necessary for blastocyst cell proliferation to continue. No such stimulatory factor has as yet been identified. In contrast, other species such as ungulates, which implant later, grow considerably before implantation. This poses the question whether the stimuli for proliferation lie within the blastocyst itself or whether soluble factors secreted by the endometrium into the uterine cavity may also play a role. Taking these observations one step back, we might ask what triggers the production of blastocyst growth promoting substances by the maternal tissues. It is possible that this stimulus originates from the embryo itself either by direct contact or by the release of embryonic specific paracrine factors, thus allowing the first recognition of the pregnant status by the mother (Dickman *et al.*, 1976).

The highest rate of placental proliferation is observed during the first trimester of human pregnancy. However, total DNA continues to increase, albeit at a slow pace, until term (Gerbie *et al.*, 1968). Since many other cells are present in the placenta, total placental growth does not necessarily reflects trophoblastic proliferation. The capacity of the CT to proliferate and form ST persists through pregnancy even if mitotic figures are difficult to detect at term. Noxious stimuli that disrupt or damage the ST under *in vivo* or *in vitro* conditions, such as low oxygen concentrations, are known to induce compensatory proliferation of the underlying CT (Fox, 1986).

The study of the mechanisms involved in the proliferation and differentiation of human trophoblasts has been hampered by the difficulty of isolating pure populations of these cells from the placenta, a complex organ formed, in addition to CT and ST, by a variety of vascular and stromal elements such as macrophages, fibroblasts and endothelial cells (Bulmer & Johnson, 1984; Enders, 1968).

Recently, several investigators have described techniques for the isolation of highly enriched populations of villous cytotrophoblasts from term and first trimester placentas. Kliman *et al.* (1987) isolated term CT and observed their differentiation *in vitro* by time lapse cinematography. CT in serum containing cultures consistently formed aggregates which subsequently transformed into syncytia within 24 to 58 hours of plating. Concomitant with syncytia formation, a progressive increase in immunocytochemical staining for SP1, HCG and HPL (in that sequential order) was observed. However, individual cells were seen to be positive for these markers at all time points, indicating that cytotrophoblasts are capable of synthesizing placental hormones. Since these authors performed their studies in the presence of 20% fetal calf serum (a known source of a variety of growth and differentiation promoting substances), the stimuli for syncytium formation was not clearly identified.

Morrish *et al.* (1986 & 1987), using serum free cultures, demonstrated that epidermal growth factor (EGF), at physiological concentrations, induced HCG and HPL secretion in isolated CT in parallel with syncytium formation. While neither of these authors observed any proliferation in their CT cultures, Yagel *et al.* (1988) was able to develop human first trimester placental cell lines by growing chorionic villous explants without enzymatic digestion. They report the establishment of several continuously proliferating primary cell lines with the morphological and biochemical characteristics of cytotrophoblasts. These lines grow in the presence of FCS, without any other exogenous growth factor, and produce HCG, progesterone and low levels of estradiol. Interestingly, while addition of exogenous HCG enhanced progesterone secretion, anti HCG antisera inhibited the proliferation of these cell lines by up to 60%, indicating a role for HCG in the autocrine regulation of CT growth. Previous studies by Goustin *et al.* (1985) had also suggested an autocrine mechanism of trophoblast proliferation involving a well known mitogen: platelet derived growth factor (PDGF), the product of the c-*sis* proto-oncogene. These authors showed by *in situ* hybridization that CT of first trimester placentas actively express c-*sis* mRNA, paralleling the distribution of c-*myc* transcripts. Furthermore, explants of first trimester chorionic villi were observed to release a PDGF-like activity in the culture medium and isolated cultured CT expressed high affinity receptors and responded to exogenous PDGF by c-*myc* activation and DNA synthesis. Quantitative determinations showed a 3-4 fold decrease in c-*sis* expression and PDGF production in the first trimester and over 10 fold decrease at term. While these studies clearly implicate PDGF as a regulatory factor in placental cell growth, other substances are likely to play a role *in vivo*, since in most instances mitogens are known to act in a coordinate and synergistic manner.

1.2 PLACENTAL FUNCTION

All of the known functions of the placenta are directed toward providing the support and protection necessary to maintain the developing embryo within the uterus. In addition to its well described metabolic functions, there is increasing evidence that the placenta is a major regulatory organ that actively coordinates countless aspects of pregnancy, from the immunological processes that prevent the fetus from rejection to the multiple hormonal mechanisms involved in normal embryonic development.

Implantation

In the development of the human blastocyst, trophoblastic cells can be distinguished from the ICM cells by the 58 cell stage (96 hours) at which time 53 cells are trophoblastic and 5 are ICM cells (reviewed in Aitken *et al.*, 1979). The first contact with the mother is made by the polar trophectoderm that at this stage has already differentiated in two layers, ST and CT. Thus, one of the earliest functions of the trophoblast is to invade the maternal tissues to allow implantation of the embryo (Fox, 1986). It is not known exactly how this process occurs nor how it is regulated to avoid the indiscriminate destruction of the endometrium but presumably proteolytic enzymes secreted by the trophoblast are involved in it. Human trophoblasts have been shown to degrade extracellular matrix (Fisher *et al.*, 1985) and production of urokinase-type plasminogen activators (uPA) by isolated term CT has recently been reported (Queenan *et al.*, 1987). cAMP analogs transiently increase uPA mRNA and protein levels but both disappear after 48 hours of culture, suggesting that other factors might be involved in the regulation of these proteases *in vivo*.

Nutrition

Mammalian embryos do not have extensive yolk stores to provide nutrients for the rapidly growing cells. Therefore, the developing fetus must receive its nutrient supply from the mother. The placenta is the specialized organ through which this is accomplished. The active selection and transfer of nutrients as well as the exchange of molecules for waste removal is perhaps the best studied of placental functions without which fetal development and growth would be impossible (Gruenwald, 1976).

Hormone Production

The recognition that the placenta is an active site of hormone synthesis and secretion is more than 80 years old (Halban, 1905). In addition to the traditional placental products such as HCG, HPL and esteroid hormones, a vast number of substances of diverse activity have been identified in conditioned medium of placental explants and the list is certainly far form complete (Chard, 1976). In fact, no other organ seems to carry out the synthesis of such diverse group of substances for such a wide range of purposes. In many instances it has not been determined whether it is the trophoblast or other stromal cells present in the placenta (macrophages, fibroblasts, endothelial cells) which secrete these substances. Furthermore, it is not known with certainty whether any of them is of crucial importance for the normal progression of pregnancy .

Human Chorionic Gonadotrophin (HCG)

The human chorionic gonadotrophin (HCG) is a 37 KD glycoprotein composed of two subunits: the alpha subunit, highly analogous to that of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), and the beta subunit that confers binding specificity (Saxena, 1983). It is now clear that the trophoblast is responsible for the synthesis and secretion of HCG as determined by immunocytochemical and *in vitro* studies on isolated CT and ST (Kliman *et al.*, 1987). The trophoblast is capable of producing HCG early during gestation; sensitive assays have been able to detect its presence as soon as 8 days after ovulation (Landesman & Saxena, 1976). Forty eight hours after implantation, the maternal blood levels of HCG start raising exponentially. Peak levels are attained between weeks 8 to 12 of gestation (160 to 500 IU/mL). Thereafter, there is a decrease in HCG levels to a plateau that is maintained throughout the remainder of pregnancy (Saxena & Landesman, 1978). Higher levels of HCG are common in multiple pregnancies (Jovanovic *et al.*, 1977) and it has been reported that many abnormal pregnancies are associated with up to 50% decrease in maternal HCG values as compared to normal gestations of the same duration (Rosal *et al.*, 1975). While the trophoblast is the main source of HCG during pregnancy, this hormone has been shown to be produced by a several other normal and neoplastic cells of diverse embryologic origin. Yoshimoto *et al.* (1979) identified substances exhibiting biologic, immunologic and physicochemical activities similar to HCG in extracts from normal kidney, lung, colon, liver, stomach and heart and suggested that the trophoblast cell is not unique in its ability to synthesize HCG but has uniquely developed the ability to glycosylate it. On the other hand, it is well recognized that abnormally high levels of HCG are present in trophoblastic tumors (Vaitukaitis & Ebersole, 1976) as well as in a variety of malignancies of non trophoblastic origin (Vaitukaitis, 1978), such as ovarian, gastric and pancreatic adenocarcinomas, hepatomas (Hung *et al.*, 1963) and germinal cell tumors of the testis (Javadpour, 1978). The regulation of the synthesis and secretion of HCG is poorly understood and appears to be different in normal trophoblasts than in trophoblastic tumors or in non-trophoblastic malignancies that secrete this hormone (Chou *et al.*, 1977; Chou, 1978). The observation that HCG-like substances are already present in sperm (Asch *et al.*, 1977) and blastocysts of rodents (Varma *et al.*, 1979) and rabbits (Zeilmaker & Verhamme, 1978) suggests that production of this hormone might be initially autonomous and determined by the embryo's own genetic program. Maternal endometrial factors might later have a role since in ectopic pregnancies, levels are normal up to day ten after ovulation and then decline to half to one third of the concentration in normal pregnancies of comparable duration (Landesman & Saxena, 1978). In this respect, it has been reported that co-cultivation of maternal lymphocytes with placental cells stimulate their secretion of HCG *in vitro* but no specific factor has as yet been identified (Dickman & Cauchi, 1978). It is well known that the alpha and beta subunits are not coordinately expressed and that the ratio of free subunits to total HCG varies during pregnancy (Vaikutaikis, 1974). At term, for example, the levels of alpha subunit exceed that of HCG. Several substances have been shown to differentially affect the secretion of HCG and its subunits in primary and transformed placental cells *in vitro*. Epidermal growth factor (Morrish *et al.*,

1987) and cAMP analogs (Feinman *et al.*, 1986) stimulate the production of HCG in term CT cells. Using temperature sensitive SV40 transformed early and term placenta cell lines Chou *et al.* (1977) observed that while sodium butyrate increased the synthesis of HCG beta, HCG alpha and HCG, Bt cAMP only stimulated the secretion of HCG alpha (Chou, 1978). Dexamethasone and cortisol greatly inhibited HCG synthesis while stimulating HCG alpha in first trimester placental lines but did not affect hormone production in term placental cells. Neither estradiol nor progesterone had any detectable effect in these systems (Mano & Chou, 1981).

The recognized biological effects of HCG are similar to those of the pituitary luteinizing hormone (LH). Both are able maintain the function of the corpus luteum of pregnancy, stimulate the release of testosterone by fetal testis and maintain the steroidogenic activity of the fetal adrenals and the placenta (Saxena, 1983). While an immunomodulatory role in the prevention of fetal rejection has also been proposed for HCG (Culouscou *et al.*, 1986), the most important function attributed to this hormone is that of the stimulation of the ovary to secrete estrogen and progesterone, steroids thought to be necessary for the growth and preparation of the endometrium for implantation (Johanssen & Bosu, 1974). Later in pregnancy, the placenta becomes autonomous in its steroid production as demonstrated by the fact that ovariectomy is well tolerated in humans after the 8th week. Trophoblastic cells have been shown to express HCG receptors and to respond to it by increased progesterone secretion; thus, autocrine stimulation of steroid production is presumably one of the mechanisms responsible for this placental autonomy. It has been debated whether HCG is absolutely necessary for the maintenance of pregnancy, since compensatory mechanisms seem to effectively operate in those cases in which low levels of this hormone are observed (Jovanovic *et al.*, 1978). However, a normal pregnancy in which production of HCG was absent has never been reported, and the measurement of HCG levels is the basis for most pregnancy tests. Moreover, immunization of marmosets and monkeys with HCG-beta has been shown to prevent pregnancy (Stevens, 1975). While these observations indicate that this hormone is somehow essential for the normal development of the embryo, the precise involvement of HCG in the many mechanisms that contribute to this process remains to be explored.

Human Placental Lactogen (HPL)

A prolactin-like activity in extracts form human placentas was first demonstrated by Ehrhardt in 1936 and finally purified by Josimovich and Mac Laren in 1962 (Josimovich & Mac Laren, 1962). This substance, which was called HPL, cross-reacted with anti-serum to human growth hormone (STH), stimulated the pigeon crop sac and induced milk formation in pseudopregnant rabbits. HPL is a single chain glycoprotein with a molecular weight of 21,000 to 23,000 daltons (Josimovich, 1981). It's amino acid composition is very similar to STH (with which it shares 168 of 190 residues) and to pituitary prolactin. HPL has been identified in the cytoplasm of ST by day 18 of gestation and in the maternal circulation by day 20 to 40. Concentrations begin to rise at about day 80 of pregnancy at the time when HCG reached its nadir and begins to decline. HPL levels continue to increase throughout pregnancy peaking at about 38 weeks when serum values are around 7-10 ug/mL. Higher levels are seen in diabetics or multiple pregnancies and decreased values have been reported in eclampsia and fetal abnormalities (Josimovich, 1981).

The regulatory mechanisms involved in HPL synthesis and secretion are not well known. It has been suggested that HPL levels depend solely on the amount of functioning trophoblastic mass (Talamantes *et al.*, 1980). In fact, maternal HPL values appear to correlate better with placental weight than with any other identifiable factor. Even pathologic pregnancies and fetal death do not cause a decline in maternal levels as long as the placenta remains healthy. Trophoblastic neoplasia, whether due to hydatidiform mole or choriocarcinoma, is characterized by low levels of HPL in serum (Josimovich, 1981). Together with the finding that HPL in trophoblast cultures declines rapidly while HCG persists, these observations suggest that some as yet unrecognized regulatory factors might specifically modulate HPL secretion. EGF (Morrish, 1987), dopamine and low calcium concentrations have been shown to stimulate the secretion of HPL *in vitro* while pimozide and high calcium seem to inhibit it. Progesterone, cortisol and insulin appear to have no effect whereas estradiol increases HPL production in early and late placental explants (Macaron *et al.*, 1978; Handwerger *et al.*, 1981).

HPL has been implicated in the preparation of mammary gland for post partum lactation, stimulation of steroidogenesis and promotion of fetal growth directly and indirectly through alteration of maternal metabolism in order to provide adequate nutrients to the fetus (Talamantes *et al.*, 1980). Among the STH-like metabolic effects attributed to HPL are the mobilization of maternal fatty acids and retention of nitrogen seen in late pregnancy (Grumbach *et al.*, 1973). Its lactogenic (Friesen, 1966) and luteotrophic (Josimovich, 1966) activities have been well documented in experimental animals. However, whether this hormone is essential for fetal survival is in doubt after the documentation by Nielsen *et al.* (1979) of a woman passing through pregnancy with no detectable levels of HPL.

Other Hormones and Pregnancy Specific Proteins

A trophoblastic specific beta globulin (pregnancy specific beta glycoprotein SP-1) appears within 18 to 23 days after ovulation but so far no hormone-like action has been associated to it (Bohn, 1971). Originally purified from human placenta, SP-1 is also present in malignant trophoblasts, breast carcinomas and malignancies of the gastrointestinal tract. Recently Heikinheimo *et al.* (1987) have identified an identical protein on the surface and cytoplasm of mature neutrophils and monocytes, the expression of which appears to increase during differentiation. The role of SP-1 in myeloid maturation remains to be established.

Several other hormone-like substances have been identified in placental explants: insulin, thyrotropin (TSH) and adrenocorticotropin (ACTH) activities (Pasqualini & Kincl, 1977) appear to be produced by this tissue in culture. The precise site of secretion of these hormones is not known and their involvement in fetal development is still speculative.

Growth Factors

Since many of the above mentioned peptides can potentially act as growth factors under certain conditions, the division between "traditional" hormones and "classical" growth factors (GF) is somehow arbitrary and only done for the sake of clarity. A number of GF-like activities have been demonstrated in human placental cell conditioned medium (HPCM). Among the best characterized are the insulin growth factors I and II (Shen *et al.*, 1986; Fant *et al.*, 1986) (IGF I and II), PDGF (Goustin *et al.*, 1985), EGF and an angiogenic factor (Moscatelli *et al.*, 1986). The presence of modulators of hemopoietic cell proliferation and function in HPCM has long been recognized (Burgess *et al.*, 1977) and factors that stimulate glial precursors (Mercanti *et al.*, 1987) and endothelial cell proliferation (Moscatelli *et al.*, 1986) have recently been reported. These findings are not surprising since placental explants contain macrophages, fibroblasts and endothelial cells, known producers of a variety of GF in other sites of the organism. The participation of the trophoblast in this activity remains to be determined. The relevance of these placental GF activities in fetal growth and development is unknown.

1.3 HEMOPOIETIC GROWTH FACTORS AND THE PLACENTA

The proliferation and differentiation of hemopoietic precursors appears to depend on the coordinated interaction of a group of cytokines whose main function is to maintain a balanced production of early undifferentiated cells and their terminally differentiated progeny (Clark & Kamen, 1987). Many of these hemopoietic growth factors (HGF) are also able to stimulate specific functions in non-proliferating mature cells. The colony-stimulating factors (CSFs), originally identified for their ability to stimulate the growth of myeloid progenitors *in vitro*, are the best characterized of the HGF.

Four CSFs have so far been purified to homogeneity and their cDNA cloned: granulocyte CSF (G-CSF), macrophage CSF (CSF-1 or M-CSF), granulocyte macrophage CSF (GM-CSF) and Multi-CSF/IL-3 (Metcalf, 1986). They are distinct glycoproteins which bind to specific receptors and differ in their actions within the hierarchical organization of hemopoietic progenitor cells and in the different cell lineages they affect. IL-3 and GM-CSF are traditionally considered pluripoietins since they seem to act on granulocytic, monocytic, megakaryocytic and erythroid cells whereas the action of G-CSF and CSF-1 is thought to be restricted to cells of the granulocytic and mononuclear phagocyte lineage respectively (Clark & Kamen, 1987). In addition to the CSFs several other cytokines have been shown to exert a modulatory action on hemopoietic cells. The specificity and function of some of them, such as eythropoietin (a key regulator in red cell maturation) (Eaves & Eaves, 1985) or interleukin-2 (Smith, 1988) (IL-2, a growth factor for T and B lymphocytes) is already well established. In other cases, such as the reported effects of the interleukins 1, 4, 6, 7, tumor necrosis factor and transforming growth factors, the precise site of action and their involvement in hemopoietic cell development *in vivo*. remains to be determined (Bartelmez & Stanley, 1985; Paul & Ohara, 1987; Billiau *et al.*, 1987; Namen *et al.*, 1988; Mizel, 1989; Branch & Guilbert, 1989).

Several types of primary human cells including fibroblasts (Tsai *et al.*, 1979), endothelial cells (Broudy *et al.*, 1986), monocytes, macrophages (Ernst *et al*, 1989) and T lymphocytes as well as many continuous cell lines of diverse embryologic origin (Demetri *et al*, 1989; Wu *et al*, 1979) produce one or more CSFs *in vitro*, either constitutively or after induction with different stimuli. However, the factors that regulate their synthesis *in vivo* are poorly understood. Bacterial lipopolysacharide (LPS), phorbol esters and monokines such as IL-1 and TNF have all been reported to increase the production of CSFs under certain conditions (Cannistra & Griffin, 1988). Some CSFs appear to be able to induce the synthesis of other CSFs (Horiguchi *et al.*, 1987) or even their own (Dinarello, 1989). The picture that emerges at this point is that the regulation of CSF production is the result of a complex network of interactions between hemopoietic and non- hemopoietic tissues that finely modulates not only the amount but the pattern of expression of these factors.

In addition to the well recognized effects of the CSFs within the hemopoietic system, several observations suggest a potential role for these factors in normal placental development (Bartocci *et al.*, 1986; Athanassakis *et al.*, 1987). Most of the evidence has been gathered in murine systems and their involvement in human pregnancy remains to be established.

Macrophage colony-stimulating factor (CSF-1)

The macrophage colony-stimulating factor (CSF-1) is a homodimeric glycoprotein that has been shown to regulate the proliferation, differentiation and survival of cells of the mononuclear phagocyte lineage (Stanley *et al.*, 1983). Immunoreactive CSF-1 has been detected in homogenates of a variety of murine tissues; normal fibroblasts, endothelial cells and macrophages secrete it in culture. Several human and murine neoplastic cell lines have been shown to synthesize CSF-1 and there is some recent evidence that normal activated B cells are able to produce the mature protein (Reisbach et al, 1989). CSF-1 levels have been shown to increase in maternal serum and tissues during murine pregnancy, particularly in the uterus, where a 10,000 fold increment in total immunoreactive protein is observed at day ten (Bartocci *et al.*, 1986). *In situ* hybridization studies have demonstrated the presence of CSF-1 mRNA transcripts in the luminal and glandular epithelium of the uterus (Regenstreif and Rossant, 1989; Pollard *et al.*, 1987, 1989). CSF-1 mRNA appears not to be restricted to the areas of direct materno-fetal contact but uniformly distributed throughout the uterus. Northern blot analysis revealed the preferential expression of a 2.3 kb CSF-1 mRNA, in contrast to other CSF-1 producing cells in which a major 4.6 kb species is usually seen. The 2.3 kb species is first detected between day 5 and 7 of pregnancy and continues to increase until day 15, coinciding with the period of maximal placental growth. Thereafter there is a relative decline but it can still be detected at term. The pregnancy-related elevation of uterine CSF-1 content can be mimicked by intravenous administration of HCG in non-pregnant mice, an effect abolished by ovariectomy suggesting that ovarian factors are key regulators of this phenomenon (Bartocci et al, 1986) . Indeed, the studies of Pollard *et al.* (1987) demonstrated that both estradiol and progesterone synergistically stimulate the secretion of CSF-1 by the endometrium. Levels comparable to those in pregnancy are only attained by the concomitant induction of a decidual reaction with arachis oil implying that other unrecognized factors might be involved in the regulation of CSF-1 synthesis. The observation that murine decidual cells are able to secrete several cytokines known to stimulate the synthesis of CSF-1 in macrophages (Guilbert *et al.*, 1990) data) could in part explain the necessity of a decidual reaction maximal for CSF-1 stimulation. The presence of CSF-1 in placental and fetal tissues is controversial. While Pollard *et al.* (1987) have not detected CSF-1 mRNA levels in those tissues, Azoulay *et al.* (1987) have observed a marked increase of CSF-1 message and protein during mouse development in all the fetal extracts examined. Highest CSF-1 protein concentrations are seen in amniotic fluid and yolk sac and peak at about day 12 of pregnancy, declining thereafter. The placenta produces low but detectable levels. No differential expression of mRNA species was observed by these authors. Interestingly, they did not detect the presence of any other CSF in fetal tissues either at the mRNA or the protein level.

CSF-1 exerts its effects on its target cells by binding to a high affinity receptor, a 165 kd transmembrane glycoprotein with tyrosine kinase activity and that is identical to the product of the c-*fms* proto-oncogene (Sherr *et al.*, 1985). The presence of CSF-1 receptors was thought to be a marker of cells of the mononuclear phagocyte lineage; however, c-*fms* transcripts have since been detected in murine and human placenta as well as in malignant trophoblast cell lines (Muller *et al.*, 1983; Rettenmeier *et al.*, 1986). Recently, the expression of c-*fms* mRNA in normal human (Hoshina *et al.*, 1985) and murine (Regenstreif and Rossant, 1989; Pollard *et al.*, 1989) trophoblasts was confirmed by *in situ* hybridization. In the mouse placenta, the highest levels of c-*fms* mRNA are observed in the giant trophoblast layer around the conceptus and appear to be developmentally regulated. All the above mentioned observations strongly indicate a potential role for CSF-1 in murine placental and/or fetal development, but evidence in this respect is only circumstantial. Athanassakis *et al.* (1987) have shown that a population of adherent placental cells of fetal origin respond to CSF-1 by increased proliferation and phagocytosis. Although these cells have not been clearly identified as trophoblasts or macrophages, both types of cells are presumably important for normal placental function.

Even less evidence exists at present of the involvement of CSF-1 in human pregnancy. While CSF-1 transcripts have been detected in term placenta and in SV-40 transformed first trimester trophoblastic cell lines (Wong *et al.*, 1987) there are no studies reported in the literature of serum or tissue concentrations of CSF-1 in pregnant women. Some interesting observations described by Maoz *et al.* (1985) could be mentioned in this context. Cyclic changes were observed in the content of blood monocytes during the menstrual cycle of normal women. The highest percentage of blood monocytes was seen during the ovulation period as well as in other conditions associated with high levels of blood estradiol. *In vitro* studies showed that estradiol at physiological concentrations increased the number of colonies developed from peripheral blood mononuclear cells whereas progesterone and testosterone had no effect. While a direct effect of estradiol on myelomonocytic precursors cannot be ruled out from these studies, an estradiol-induced increase in CSF-1 production by bone marrow, spleen or even endometrial cells might in part account for this phenomenon. This poses the intriguing question whether the elevation of maternal CSF-1 levels is an event prior to implantation or even fertilization and might somehow condition the normal development of these very early processes of pregnancy. Human trophoblasts have been shown to express c-*fms* mRNA

(Hoshina *et al.*, 1985), thus they are potential targets for CSF-1 stimulation. In addition to its proliferative capacity, CSF-1 broadly influences the function of mature mononuclear phagocytes by stimulating their production of diverse cytokines (Warren & Ralph, 1988), prostaglandins (Kurkland *et al.*, 1979), oxygen products (Wing *et al.*, 1985) and plasminogen activators (Lin & Gordon, 1979) among other pleiotropic responses; therefore, the effects of CSF-1 on placental trophoblasts should be sought in a variety of conditions. Does CSF-1 regulate the growth and/or the secretion of hormones in human trophoblasts? Does it stimulate their production of proteolytic enzymes to facilitate the implantation of the embryo? Does it increase the microbicidal properties of the trophoblast, thus preventing pregnancy losses due to fetal infection? Does CSF-1 have different functions at different times of gestation? The availability of pure populations of human trophoblasts (villous and extravillous) should help to answer some of these questions and as a result define the precise role of CSF-1 in fetal development.

Granulocyte Macrophage Colony-stimulating Factor (GM-CSF)

Human GM-CSF is a 22,000 dalton single chain glycoprotein ,first purified by Gasson *et al.* (1984) from conditioned medium of a human T cell leukemia cell line (Golde *et al.*, 1978). It has been shown to be synthesized by normal T cells, fibroblasts, macrophages and endothelial cells (Metcalf, 1986) but only after induction with diverse stimuli (Thorens *et al.*, 1987). Several malignant cells lines produce it constitutively and GM-CSF transcripts have been detected in primary acute myeloid leukemia blasts (Young *et al.*, 1987).

GM-CSF also regulates several functions of mature granulocytes (Weisbart *et al.*, 1985), monocytes (Grabstein *et al.*, 1986) and eosinophils (Silberstein *et al.*, 1986) through the binding to a specific 84,000 da high affinity receptor expressed by these cells (Dipersio *et al.* , 1988). Functional GM-CSF receptors have been recently identified in malignant cell lines of non-hemopoietic origin (Baldwin *et al.*, 1989) suggesting that this CSF might have a broader range of specificities than originally suspected. Human placental cell conditioned medium (HPCM) has been long recognized as a source of hemopoietic colony-stimulating activity (CSA) (Burgess *et al.*, 1977). Recently, Cukrova and Hrkal (1987) have isolated a molecule biochemically indistinguishable from GM-CSF from term placental explants. Whether this molecule is produced by trophoblasts or villi stromal cells has not been determined, nor have the factors that regulate its expression. The involvement of GM-CSF in placental development is even more obscure. While Athanassakis *et al.* (1987) have demonstrated a stimulatory effect of GM-CSF on murine placental cells, other authors have reported that high concentrations of this factor inhibit mouse blastocyst development *in vitro* (Hill *et al.*, 1987). GM-CSF receptors have been identified in human placental membranes and malignant trophoblasts (Scheffler *et al.*, 1989) and the receptor has recently been cloned from a placental cDNA library (Gearing *et al.*, 1989), but so far effects of this CSF on trophoblast growth or function have not been reported. GM-CSF is known to synergize with CSF-1 (Guilbert, 1985; Guilbert and Branch, 1989) and induce its synthesis by mononuclear phagocytes (Horiguchi *et al.*, 1987) as well as to mimic many of the functions of CSF-1, thus the coordinated interaction of both factors could potentially be implicated in the regulation of the diverse placental functions.

Other Hemopoietic Growth Factors

Main *et al.* (1987) have recently demonstrated the presence of interleukin-1 in purified term CT. LPS greatly enhances the production of IL-1 in these cells and the levels attained are similar to those of peripheral blood monocytes on a cell to cell basis. Given the pleiotropic effects of IL-1 in other systems, these authors speculate that IL-1 may regulate protease production as well as determine uterine vasculature changes, both phenomena important during embryo implantation.

Several lymphokine-like activities have been detected in human placental explant supernatants, the precise nature of which remains to be determined. In this respect, *in situ* hybridization studies have shown the presence of interleukin-2 transcripts in human syncytiotrophoblast (Bohem *et al.*, 1989), a most intriguing finding since this cytokine was thought to be a marker of cells of the lymphocytic lineage. These authors did not observe any correlation between the level of IL-2 mRNA expression and the stage of pregnancy. They suggest that trophoblast derived IL-2 could be involved in local immunomodulation, acting on uterine IL-2 receptor bearing cells.

While the evidence at present is in many cases only circumstantial, all these exciting observations imply that a network of related hematopoietic cytokines might directly influence the initiation and maintenance of normal pregnancy, establishing unsuspected links between the hemopoietic and the reproductive systems. Future research should help to clarify the role of the trophoblast itself as a key intermediary is this phenomenon as well as to determine the precise site of action, the function and the regulation of hematopoietic cytokines in fetal and maternal tissues. Furthermore, the comprehension of the role the various hematopoietic cytokines in human pregnancy might contribute to the understanding of the causes underlying disturbances of reproduction such as infertility, abortion, intrauterine growth retardation and trophoblastic malignancies.

1.4 PLACENTAL PATHOLOGY

The spectrum of pathological disturbances of the placenta includes both structural and functional alterations, infections and tumors of trophoblastic and non trophoblastic origin. Given its multiple involvement in normal pregnancy, the placenta has also been implicated in a variety of disturbances of reproduction such as eclampsia, abortion and fetal intrauterine growth retardation (Fox, 1986).

The macroscopic lesions of the placenta have been extensively described in the literature as well as diverse histological abnormalities of the development and maturation of chorionic villi structures. While most of the visible alterations of the placenta are of no functional significance, a failure in the maturation of the villous tree may impair the functional efficiency of this organ, as may defective trophoblast differentiation (Fox, 1986). The impact of these abnormalities on fetal growth remains to be established as do the pathologic mechanisms that provoke them.

Placental Insufficiency

It is becoming increasingly evident that the cause underlying most cases of presumed "placental insufficiency", an ill defined entity assumed to be one of the causes of fetal intrauterine growth retardation, is a reduced maternal blood flow to the feto-placental unit (Gruenwald *et al.*, 1976), resulting from the partial or complete failure of the extravillous trophoblast to invade the intramyometrial segments of the uterine spiral arteries early during gestation (Pijneborg *et al.*, 1981). Since the factors that control normal trophoblast invasion are unknown, no molecular explanation for this pathological phenomenon exists at present.

Abortion

Abortion, as defined in practical terms, is any pregnancy that terminates before the fetus is viable. It has been suggested that more than 45% of all pregnancies end in abortion, many of them unnoticed. There are some well recognized causes of abortion such as uterine malformations or severe systemic maternal diseases but in most cases the etiology is unknown (Fox, 1978). Embryonic chromosomal abnormalities have been thought in the past to be the main cause of abortion: however, chromosomal abnormalities are also seen in fetuses developed in otherwise uneventful pregnancies. The hypothesis that abortion can in some instances be caused by an immune mediated graft rejection process has received much support, but there is no conclusive evidence in this respect (Redman, 1986). In fact, whether immune suppressive or stimulating mechanisms favor the maintenance of pregnancy is controversial (Wegmann, 1984). The involvement of the placenta in the pathogenesis of abortion has not been proved. Alterations in trophoblast implantation, proliferation and/or expression of its differentiated functions could all potentially lead to an aborted pregnancy.

Trophoblastic Tumors

Three distinct entities are comprised in the spectrum of gestational trophoblastic disease (GTD): hyatidiform mole, invasive mole or chorioadenoma destruens and choriocarcinoma. The latter is by clinical and histological parameters the only true malignancy of the trophoblast with an overall mortality of over 80% in untreated cases (Hertz, 1978). Hydatidiform mole (HM) is included in this classification due to its close association with choriocarcinoma (CC) of which it is considered a pre-malignant state since CC are preceded by HM in up to 50% of the cases (Bagshawe, 1976a). A HM is considered to be invasive when molar villi have penetrated into the myometrium or its blood vessels. Invasive moles are able to metastasize but the mortality associated with this entity is due more to hemorrhagic events than to uncontrollable spread of neoplastic cells (Park, 1971). At present, evidence is lacking that patients with invasive moles are more likely to develop CC than those with HM.

GTD is at least ten times more frequent in Third World countries than in Europe or North America (Joint Project, 1969). This remarkable variation in geographical distribution led to the suggestion that nutritional and genetic factors such as thymidine deficiency or consanguinity might be implicated in the pathogenesis on the disease, a hypothesis that so far has not been proved.

Hydatidiform Mole

Complete HM is an abnormal pregnancy histologically characterized by grossly swollen villi in the absence of a fetus. Recent cytogenetic studies demonstrated that complete HM is androgenetic in origin: the entire genome of the molar conceptus is paternally derived (Wake *et al.*, 1978). More than 90% of the cases result from the fertilization of an empty egg (with a blighted or absent nucleus) by an haploid sperm that then duplicates without cytokinesis and restores diploydy (monospermic moles). Fertilization of an empty egg by two spermatozoids accounts for the remaining 10% of cases (dyspermic moles) (Ohama *et al.*, 1981). Monospermic moles are invariably homozygous whereas dyspermic moles may be heterozygous for a given genetic marker. HM is an essentially benign condition; however, a molar pregnancy may precede the development of an overt choriocarcinoma in about 3% of the cases. Heterozygous moles appear to be more prone to malignization than homozygous moles (Wake *et al.*, 1984), an observation further substantiated by the fact that all the invasive moles and choriocarcinomas studied by Wake *et al.* (1984) were of dyspermic origin. The factors underlying this selective transformation of heterozygous moles remain to be determined.

Choriocarcinoma

Choriocarcinoma can develop after any type of normal pregnancy: normal, abortion, molar or ectopic. It is a highly malignant tumor whose poor prognosis has been dramatically changed by the introduction cytotoxic agents. Subsequent normal gestations have been reported after successful treatment with anti-folate drugs. However, about 20% of the cases are still refractory to therapy (Goldstein & Berkowitz, 1982).

Histologically, CC are invariably composed by both syncytio and cytotrophoblastic cells but organized chorionic villi are never seen. Morphological diagnosis is sometimes difficult since the overall microscopical structure of CC recapitulates that of the normal trophoblast of the early implantation blastocyst (Gore & Hertig, 1970).

CC characteristically secrete high levels of chorionic gonadotrophin and the amount of trophoblast proliferation can be approximately estimated from the values of HCG excretion (Bagshawe, 1976b). Determinations of serum and urine levels of HCG are used not only for the diagnosis but for the monitoring of CC patients during and after treatment. In contrast, the concentration of human placental lactogen in serum in CC are usually low. This suggests that factors other than total trophoblastic mass determine the amount and pattern of hormone production in trophoblastic malignancies. Evidence for an altered regulation of HCG synthesis has been presented by Vaitukaitis and Ebersole (1976) who showed that patients with GST have widely different rates of HCG and its subunits in serum and tumor extracts.

Because of its rarity and the decrease of major surgery for the condition, material from CC has become less widely available for study. In 1966 Patillo and Gey (1968) developed a continuous cell line derived from a primary choriocarcinoma which had been serially transplanted into hamster cheek pouch by Hertz (1959). This pure cytotrophoblastic culture called BeWo was the first hormone synthesizing culture system to be established in continuous cultivation.

Several other CC cell lines have been developed since then, and have been widely used as models in the investigation of mechanisms of cell differentiation, hormone production and drug cytotoxicity.

Pathogenesis of Trophoblastic Tumors

Despite extensive research, the precise cause of CC, like that of many other cancers, remains obscure. The peculiar geographical association of this tumor has prompted some authors to suggest that racial, nutritional or viral mechanisms are involved in its pathogenesis, but so far this has not been proven.

Overexpression of normal proto-oncogenes and/or their abnormal activation through mutational or combinatorial events are mechanisms implicated in the genesis of cancer cells (Bishop, 1987). No transforming activity has been reported to be present in primary malignant trophoblasts. Choriocarcinoma cell lines do express c-*fms* mRNA but its protein product appears to be biochemically and structurally identical to that expressed by normal monocytes (Woolford *et al.*, 1985; Rettenmeier *et al.*, 1986).

One of the mechanisms by which oncogenes are thought to be able to transform normal cells is by diminishing the requirements for exogenous growth factors. Autonomous growth can be achieved by alterations at the receptor, transducer or effecter levels of the mitogenic signal (Goustin *et al.*, 1986). For example, mutations at specific sites of the CSF-1 receptor protein confer the receptor the ability to permanently originate a proliferative signal in murine NIH 3T3 cells, independent of CSF-1 activation (Roussel *et al.*, 1988). Similar observations have been made with another growth factor receptor of the tyrosine-kinase family, namely the epidermal growth factor receptor (Carpenter, 1987).

Another mechanism whereby normal cells can become autonomous is by developing the ability to synthesize their own stimulatory molecules, a process globally known as autocrine stimulation (Sporn & Todaro, 1980). Autocrine growth stimulation is thought to provide the cells with selective proliferative advantage by short circuiting mechanisms involved in the normal regulation of cellular growth. Autocrine growth is not, however, a unique property of tumor cells and it may occur in conditions requiring extraordinary proliferative demands such as embryogenesis. There is some evidence that normal trophoblast growth is regulated by platelet derived growth factor (Goustin *et al.*, 1985) and HCG (Yagel *et al.*, 1988) in an autocrine manner. Unknown is whether quantitative alterations in their expression can lead to trophoblast transformation or whether other autocrine factors can play a role in this phenomenon.

It is generally thought that tumors arise from a multistep mechanism whereby a cell acquires the capacity for extended proliferation, invasion and metastasis (Land *et al.*, 1983). The common view is that each step generates additional genotypic or phenotypic alterations that eventually lead to the fully transformed state. In this context, choriocarcinomas could provide a suitable model for the study of tumor progression . By elucidating the mechanisms that give rise to dyspermic moles and following the genetic and/or biochemical changes that accompany their progression into choriocarcinomas, much insight could be gained not only about the malignant transformation of human trophoblasts but also about the pathogenesis of neoplasia in general.

1.5 CONCLUSIONS

The placenta, a complex multifunctional organ interposed between the maternal and fetal tissues is bound to play a crucial role in the initiation and maintenance of normal pregnancy. However, the molecular mechanisms that regulate its development and activity are poorly understood. The role ascribed to traditional pregnancy associated hormones such as steroids, chorionic gonadotrophin and placental lactogen will have to be revised on the light of recent discoveries that link other previously unrelated general regulators to the process of normal reproduction. Several cytokines and some of their receptors has been detected in the placenta or in its vicinity. Whether these observations represent true developmental mechanisms remains to be established.

Evidence is being accumulated from several laboratories that the colony-stimulating factors (CSFs), whose function was thought to be restricted to cells of hemopoietic origin, may also play a role during normal pregnancy. While the site of synthesis, the targets and the function of the CSFs in fetal development is still speculative, the pioneering work of Pollard and his coworkers, as well as that of Tom Wegmann's group in the murine model, strongly suggest a physiologic association of these factors with placental function.

During the formation of the placenta, trophoblast, its most specialized tissue, proliferates, differentiates, invades maternal structures and secretes a variety of substances thought to be essential for the maintenance of pregnancy. It selectively transfers oxygen and nutrients to the fetus and at the same time protects the conceptus from harmful immune reactions as well as from microbial infection. The potential sites for regulation by the CSFs (themselves involved at many stages of myeloid proliferation and differentiation) in this vast spectrum of trophoblast activities are innumerable and deserve to be investigated.

It is well established that the syncytiotrophoblast, the main site of synthesis of placental hormones, arises from the differentiation of underlying cytotrophoblastic cells whereas the origin and function the different extravillous trophoblasts is still unclear. Thus, generalizations about trophoblast regulatory mechanisms might be misleading and must be examined for each specific subset of cells.

To be able to answer all the questions that exist at present about placental development is clearly beyond the scope of this thesis . Thus I have focused in two in two general but essential questions:

1. Do normal trophoblasts express functional receptors for hemopoietic growth factors? If so, do they respond to them in any manner?
2. If hemopoietic growth factors are involved in the proliferation and/or differentiation of normal human trophoblasts, can an aberrant expression and/or regulation of these factors or their receptors be implicated in the pathogenesis of choriocarcinomas?

1.6 RESEARCH PROPOSAL & EXPERIMENTAL APPROACH

I have chosen to study in depth the effects of two myeloid HGFs: CSF-1 and GM-CSF, on placental trophoblasts *in vitro*. The former, because of the existing evidence in the murine model; the latter because of its recognized overlapping functions and synergistic effects with CSF-1 on mononuclear phagocytes.

Term villous trophoblasts were used as a prototype of normal trophoblast, given their accessibility and the existence of efficient methods for their purification and characterization.

While GM-CSF receptors have been detected in placental membranes and choriocarcinoma cell lines, there was no evidence for the presence of CSF-1 receptors in normal human trophoblasts. Thus I first assessed the expression of CSF-1 R transcripts in purified term CT by Northern analysis and confirmed its presence at the cell surface by radio-labeled CSF-1 binding assays.

In this arguably oversimplified model of humoral-cellular interactions, I next addressed two specific questions:

1. Do GM-CSF and CSF-1 stimulate the growth of cytotrophoblast cells?
2. Alternatively, do these factors selectively inhibit the proliferation of CT by inducing their terminal differentiation into syncytiotrophoblasts?

Variations in the incorporation of ^{3}H thymidine into CT after co-incubation with the specified CSFs were determined as a sign of their effects on CT proliferation, whereas morphological changes and increased HCG and HPL secretion were assessed as indicators of CT differentiation.

In the investigation of the possible role of CSF-1 and GM-CSF as autocrine growth factors in trophoblastic malignancies, three well characterized choriocarcinoma cell lines (BeWo, JEG-3 and JAR) were used as a model of the fully transformed state, since no primary tissue could be obtained at the time of these studies.

Autocrine growth is by definition a process of self stimulation that occurs in those cells that produce a certain factor for which they have specific receptors and are able to respond. These three aspects of the autocrine model were examined in the CC cell lines.

First I determined whether CC produced CSF-1 and/or GM-CSF in culture. For that purpose assays that could specifically quantitate these CSFs were developed. After establishing that the three cell lines expressed cell surface receptors for CSF-1, I examined the effects of both factors on cell proliferation. Comparative studies were also performed with various other cytokines. The fact that an autonomously growing cell both produces and responds to a given growth factor does not necessarily mean that autocrine stimulation is directly responsible for this autonomy. To further confirm my original hypothesis, I employed neutralizing antibodies to GM-CSF and to the CSF-1 receptor in an attempt to inhibit the spontaneous proliferation of the CC.

My results are presented in the form of two principal papers, the first describing the effects of CSFs on normal trophoblasts and the second the evidence obtained using the CC cell lines and a final discussion linking these two studies is also included.

BIBLIOGRAPHY

Aitken R.J., Beaconsfield R. and Ginsburg J. Origin and formation of the placenta. In: Placenta - A neglected experimental model. (P. Beaconsfield and P. Villee, eds.) Pergamon Press, 1979. pp 152-163.

Asch R.H., Fernandez C.O. and Pauerstein C.J. Immunodetection of human chorionic gonadotrophin-like substance in human sperm. Fertil. Steril. 28:1258-1260, 1977.

Athanassakis I., Bleackley C.R., Paetkau V., Guilbert L.J., Barr P.J. and Wegmann T.G. The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells J. Immunol. 138:37-44, 1987.

Azoulay M., Webb C.G. and Sachs L. Control of hematopoietic cell growth regulators during mouse fetal development. Mol. Cell. Biol. 7:3361-3363, 1987.

Bagshawe K.D. Risk and prognostic factors in trophoblastic neoplasia. Cancer 38:1373-1385, 1976a.

Bagshawe K.D. Endocrinology and the treatment of trophoblastic tumors. J. Clin. Pathol 29:140-149, 1976b.

Baldwin C.C.G., Gasson J.C., Kaufman S., Quan S., Williams R.E., Avalos B., Gazdar A.F., Golde D.W. and DiPersio J.F. Non-hematopoietic tumor cells express functional GM-CSF receptors. Blood 73:1033-1037, 1989.

Bartelmez S.H. and Stanley E.R. Synergism between hemopoietic growth factors detected by their effects on cells bearing receptors for a lineage-specific hemopoietic growth factor. Assay for hemopoietin 1. J. Cell. Physiol. 122:370-375, 1985.

Bartocci A., Mastrogiannis D.S., Migliorati G., Stockert R.J., Wolkoff A.W. and Stanley E.R. Regulation of colony-stimulating factor-1 during pregnancy. J. Exp. Med. 164:956-961, 1986.

Beaconsfield P., Birdwood G. and Beaconsfield R. The Placenta. Sci. American 243:95-102, 1980.

Billiau A., Van Damme J., Ceuppens J. and Baroja M. Interleukin-6, a ubiquitous cytokine with paracrine as well as endocrine functions. In: Lymphokine Receptor interactions (D. Fradelizi and J. Bertoglio, eds.) London: John Libby Eurotext Ltd, 1987. pp 133-142.

Bishop J.M. The molecular genetics of cancer. Science 235:305-311, 1987.

Bohem K.D., Kelley M.F., Ilan J. and Ilan J. The interleukin-2 gene is expressed in the syncytiotrophoblast of the human placenta. Proc. Natl. Acad. Sci. (USA) 86:656-660, 1989.

Bohn H. Detection and characterization of pregnancy specific proteins in the human placenta and their quantitative immunochemical determination in sera from pregnant women. Arch. Gynek. 210:440, 1971.

Boyd J.D. and Hamilton W.J. The human placenta. Mac Millan, 1974.

Branch D.R. and Guilbert L.J. Synergistic stimulation of macrophage proliferation by the monokines tumor necrosis factor alpha (TNF) and colony-stimulating factor-1 (CSF-1). Blood 73:307-311, 1989.

Broudy V.C., Kaushansky K., Segal J.M., Harlan J.M. and Adamson J.W. Tumor necrosis factor alpha stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. (USA) 83:7467-7470, 1986.

Bulmer J.N. and Sutherland C.A. Bone marrow origin of endometrial granulocytes in the early human placental bed. J. Rep. Immunol. 5:383-387, 1983.

Bulmer J.N. and Johnson P.M. Macrophage populations of the human placenta and amniochorion. Clin. Exp. Immunol. 57:393-403, 1984.

Burgess A.W., Wilson E.M.A. and Metcalf D. Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood 49:573-583, 1977.

Cannistra S.A. and Griffin J.D. Regulation of the production and function of granulocytes and macrophages. Semin. Hematol. 25:173-181, 1988.

Carpenter G. Polypeptide growth factors receptors. Ann Rev Biochem 56:881-902, 1987.

Chard T. Normality and abnormality. In: Placental hormone assays in the evaluation of fetal well-being. (A. Klopper, ed.) Edinburgh: Churchill Livingston , 1976.

Chou J.C. Establishement of clonal human placental cells synthesizing human chorionic gonadotrophin. Proc. Natl. Acad. Sci. (USA) 75:1854-1858, 1978.

Chou J.C., Robinson J.C. and Wang S.S. Effects of sodium butyrate on synthesis of human chorionic gonadotrophin in trophoblastic and non-trophoblastic tumors. Nature 268:543-544, 1977.

Clark S.C. and Kamen R. The human hemopoietic colony-stimulating factors. Science 236:1229-1237, 1987.

Cukrova V. and Hrkal Z. Purification and characterization of granulocyte-macrophage colony-stimulating factor from human placenta. J. Chromatogr. 413:242-246, 1987.

Culouscou J.M., Remacle-Bonnet M.M. Pommier G., Rance R.J. and Depieds R.C. Immunosuppresive porperties of the human placenta: study from supernatants from short term syncytiotrophoblast cultures. J. Reprod. Immunol. 9:33, 1986.

Demetri G.D., Zenzie B.W., Rheinwald J.G. and Griggin J.D. Expression of colony-stimulating factor genes by normal and malignant mesothelioma cell lines *in vitro*. Blood 74:940-946, 1989.

Dickman Z., Dey S.K. and Gupta J.S. A new concept: control of early pregnancy by hormones originating in the preimplantation embryo. Vitam. Horm. 34:215-242, 1976.

Dickman W.J. and Cauchi M.N. Lymphocyte-induced stimulation of human chorionic gonadotrophin production by trophoblastic cells *in vitro*. Nature 271:377-378, 1978.

Dipersio J.F., Billing P., Kauffman S., Eghtesady P., Williams R.E. and Gasson J.C. Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. J. Biol. Chem. 263:1834, 1988.

Eaves A.C. and Eaves C.J. Erythropoiesis in culture. In: Clinics in Hematology (E.A. Mc Culloch, ed.) Toronto: W.B. Sanders, 1985. pp 371.

Enders A.C. Fine structure of anchoring villi of the human placenta. Am. J. Anat. 122:419-451, 1968.

Ernst T.J., Ritchie A.R., Demetri G.D. and Griffin J.D. Regulation of granulocyte and monocyte colony-stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post-transcriptional level. J. Biol Chem. 264:2700, 1989.

Fant M., Munro H. and Moses A.C. An autocrine/paracrine role for insulin growth factors in the regulation of human placental growth. J. Clin. Endoc. Metab. 63:499-505, 1986.

Feinman M.A., Kliman H.J. and Strauss J.F. 8-bromo 3',5' adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. J. Clin. Endoc. Metab. 63:1211-1217, 1986.

Fisher S.J., Leitch M.S., Kantor M.S. and Kramer R.H. Degradation of extracellular matrix by the trophoblastic cells of first trimester human placentas. J. Cell. Biochem. 27:31-41, 1985.

Fox H. The placenta in abortion. In: Pathology of the placenta (H.H. Fox and J.L. Bennington, eds.) W.B. Saunders, 1978. pp 258-269.

Fox H. Pathology of the placenta. Clinics in Obstetrics and Gynecology 13:501-520, 1986.

Friesen H. Lactation induction by human placental lactogen and cortisone acetate in rabbits. Endocrinology 79:212-216, 1966.

Gardner R.L. and Rossant J. Determinatioon during embryogenesis. In: Embryogenesis in mammals. Ciba Found. Symp. 40. Elseevier: North Holland Publishers, 1976. pp 5-18.

Gasson J.C., Weisbart R.H., Kaufman S.E., Clark S.C., Hewick R.M., Wong G.G. and Golde D.W. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. Science 226:1339. 1984.

Gerbie A.B., Hathaway H.H. and Brewer J.I. Autoradiographic analysis of normal trophoblast proliferation. Am. J. Obst. Gynec. 100:640-650, 1968.

Glasser S.R. and Mc Cromack S.A. Cellular and molecular aspects of decidualization and implantation. In: Proteins and Steroids in early mammalian development. (H. Beier and P. Karlson, eds.) Springer Verlag, 1980.

Golde D.W., Quan S.G. and Cline M.G. Human T lymphocyte cell line producing colony-stimulating activity. Blood 52:1068, 1978.

Goldstein D.P. and Berkowitz R.S. Gestational trophobalstic neoplasms. Clinical principles of diagnosis and management. Philadelphia: W.B. Saunders, 1982. pp 188-288.

Gore H. and Hertig A.T. Problems in the histologic interpretation of the trophoblast. Clinics in Obstetrics and Gynecology 10: 269, 1970.

Goustin S.A., Bertzholtz C., Pfeiffer-Ohlsson S., Persson H., Rydnert J., Bywater M., Holmren G., Heldin C., Westermark B. and Ohlsson R. Coexpression of the sis and myc proto-oncogenes in developing human placenta suggests autocrine control of trophoblast proliferation. Cell 41:301-312, 1985.

Grabstein K.H., Urdal D.L., Tushinski R.J., Mochizuki D.Y., Price V.L., Cantrell M.A., Gillis S. and Conlon P.J. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science 232:506-508, 1986.

Gruenwald P. The placenta and its maternal supply line (P. Gruenwald, ed.) Baltimore: University Park Press, 1976. pp 1-12.

Grumbach M.M., Kaplan S.L. and Vinik A. Human chorionic somatotrophin. In: Methods in investigative and diagnostic endocrinology. (S.A. Berson and R.A. Yalus, eds.) Amsterdam: North Holland Publishers, 1973.

Halban J. Die imeresekrétion von ovarium und placenta und ihre bedeutung fur die function der milchendere. Arch. Gynek. 75:353, 1905.

Handwerger S., Conn P.M., Barrett J. and Golander A. Human placental lactogen release *in vitro*: paradoxical effects of calcium. Am. J. Physiol. 240:E550, 1981.

Heikinheimo M., Gahmberg C.G., Bohn H. and Anderson L.C. Oncoplacental protein SP1-A constitutive and inducible late differentiation marker of the human myelomonocytic lineage. Blood 70:1279-1283, 1987.

Hertz R. Choriocarcinoma of women maintained in serial passage in hamster and rat. Proc. Soc. Exp Biol. Med. 102:77-80, 1959.

Hertz R. Spectrum of gestational trophoblastic disease. In: Choriocarcinoma and related gestational trophoblastic tumors in woman. New York: Raven Press. 1978, pp 13-22.

Hill J.A., Himovici F and Anderson D.J. Products of activated lymphocytes and macrophages inhibit mouse embryo development *in vitro*. J. Immunol. 139:2258-2254, 1987.

Horiguchi J., Warren M.K. and Kufe D. Expression of macrophage colony-stimulating factor-1 (CSF-1) in human monocytes treated with granulocyte-macrophage colony-stimulating factor. Blood 69:1259-1261, 1987.

Hoshina M., Nishio A., Bo M., Boime I. and Mochizuki M. The expression of the oncogene fms in human chorionic tissue. Acta Obstet. Gynec. (Japan) 37:2791-2798, 1985.

Hung W., Blizzard R.M., Migeon C.J., Camacho A.M. and Nyhan W.L. Precocious puberty in a boy with hepatoma and circulatory gonadotrophin. J. Pediatr. 63:895, 1963.

Inskeep E.K., Wilson L., Butler W.R., Dierschke D.J., Fritz G.R. and Knobil E. Endometrila secretion of prostaglandin during the ovarian cycle and early pregnancy. In: The Endometrium (F.A. Kimball, ed.) SSP Medical and Scientific Books, 1983, pp 287-310.

Javadpour J. Current concepts in management of testicular tumor. Urol. Survey 28:89-96, 1978.

Johanssen E.D., Bosu W.T.K. The function of the corpus luteum in primates. In: Physiology and genetics of reproduction (E.M. Cpountinho and F. Fuchs, eds.) New York: Plenum Press, 1974. pp 343-352.

Joint Projects for study of choriocarcinoma and hydatidiform mole in Asia. Geographic variation in the occurrence of hydatidiform mole and choriocarcinoma. Ann. N.Y. Acad. Sci. 80:178-195, 1969.

Josimovich J.B. Maintenance of pregnancy in the rat by synergism between human placental lactogen and hCG. Endocrinology 83:707, 1966.

Josimovich J.B. Placental lactogen and pituitary prolactin. In: Endocrinology of pregnancy. (A. Klopper and F. Fuchs, eds.) Philadelphia: Harper and Row, 1981. pp 144-160.

Josimovich J.B. and Mac Laren J.A. Presence in the human placenta and term serum of a highly lactogenic substance, immunologically related to pituitary growth hormone. Endocrinology 71:209-214, 1962.

Jovanovic I., Landesman R. and Saxena B.B. Screening for twin pregnancy. Science 198:738-749, 1977.

Jovanovic I., Dawood M.Y. and Saxena B.B. Hormonal profile as a prognostic index of early threatened abortion. Am. J. Obstet. Gynecol. 130:274-278, 1978.

Kaufmann P. and King B.F. Structural and functional organization of the placenta. Basel: S. Karger. 1982.

Kearns M. and Lala P.K. Bone marrow origin of decidual cell precursor in the pseudopregnant rat uterus. J. Exp. Med. 155-1537, 1982.

Kliman H., Feinman M.A. and Strauss J.F.III Differentiation of human cytotrophoblasts in syncytiotrophoblasts in culture. Troph. Research 2:407-421, 1987.

Kurkland J.I., Piles L.M., Ralph P., Bockmann R.S. and Moore M.A.S. Induction of prostaglandin E in normal and neoplastic macrophages. Role of colony-stimulating factor(s) distinct from effects on myeloid cell proliferation. Proc. Natl. Acad. Sci. (USA) 76:2326-2330, 1979.

Land H., Parada L.F. and Weinberg R.A. Cellular oncogenes and multistep carcinogenesis. Science 222:771-777, 1983.

Landesman R. and Saxena B.B. Results of the first 1000 radioreceptor assays for the determination of human chorionic gonadotrophin... A new, rapid, reliable and sensitive pregnancy test. Fertil. Steril. 27:357-362, 1976.

Landesman R. and Saxena B.B. Diagnosis of ectopic pregnancy by radioreceptor assay for chorionic gonadotrophin. Lancet 8067:772-774, 1978.

Lin H.S. and Gordon S. Secretion of plasminogen activator by bone marrow dereived mononuclear phagocytes and ites enhancement by colony-stimulating factor. J. Exp. Med. 150:231-245, 1979.

Macaron C., Famuyiwa O. and Singh S.P. *In vitro* effect of dopamine and pimozide on human chorionic somatomammotrophin (hCS) secretion. J. Clin. Endoc. Metab. 47:168, 1978.

Main E.K., Strizki J. and Schochet P. Placental production of immunoregulatory factors: Trophoblast is a source of interleukin-1. Trophoblast Research 2:149-160, 1987.

Mano T. and Chou J.C. Regulation of human chorionic gonadotrophin synthesis in cultured human placental cells by glucocorticoids. Endocrinology 109:888-892, 1981.

Maoz H., Kaiser N., Halimi M., Barak V., Haimovits A., Weinstein D., Simon A., Yagel S., Biran S. and Treves A. The effect of estradiol on human myelomonocytic cells I. Enhancement of colony formation. J. Reprod. Immunol. 7:325-335, 1985.

Martin L. and Finn C.A. Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus. J. Endoc. 41:363-371, 1968.

Mc Cormack S. and Glasser S.R. Hormone production by rat blastocysts and mid-gestation trophoblast *in vitro*. In: The Endometrium (F.A. Kimball, ed.) SPP Medical and Scientific Books, 1983. pp 145-190.

Mercanti D., Luzzatto E., Ciotti M.T. and Levi G. Mitogenic effect of a human placental factor on astrocytes and glial precursors. Exp. Cell Res. 168:182-190, 1987.

Metcalf D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood 67:257-263, 1986.

Mizel S.B. The interleukins. FASEB J. 3:2379-2388, 1989.

Morrish D.W., Bhardwaj D., Dabbagh L.K., Marusyk H. and Siy O. Epdermal growth factor induces the differentiation and secretion of human chorionic gonadotrophin in normal human placenta. J. Clin. Endoc. Metab. 65:1282-1290, 1987.

Moscatelli D.A., Presta M., Mignatti P., Mullins D.E., Crowe R.M. and Rifkin D.B. Purification and biological activities of an angiogenesis factor from human placenta. Anticancer Research. 6:861-863, 1986.

Moscatelli D., Presta M. and Rifkin D.B. Purification of a factor from human placenta that stimulates capillary endothelial cells protease production, DNA synthesis and migration. Proc. Natl. Acad. Sci. (USA) 83:2091-2095, 1986.

Muller R., Slamon D.J., Adamson E.D., Tremblay J.M., Muller D., Cline M.J. and Verma I.M. Transcription of c-*onc* genes c-*ras* and c-*fms* during mouse developement. Mol. Cell. Biol. 3:1062-1069, 1983.

Namen A.E., Lupton S., Hjerrild K., Wignall J., Schmierer A., Mosley B., March C.J., Urdal D., Gillis S., Cosman D. and Goodwin R.G. Stimulation of B cell progenitors by cloned murine interleukin-7. Nature 333:571-573, 1988.

Nehemiah J.E., Schnitzer J.A, Schulman H. and Novikoff A.B. Human chorionic trophoblasts, decidual cells and macrophages: histochemical and electron microscopic study. Am. J. Obstet. Gynecol. 140:261-26, 1981.

Nielsen P.V., Pedersen H. and Kampmann E.M. Absence of human placental lactogen in an otherwise uneventful pregnancy. Am. J. Obstet. Gynec. 135:322-324, 1979.

Ohama K., Kajii T., Okamoto E., Fujuda Y., Imaizmi K., Tsukahara M., Kobayashi K. and Hagiwara K. Dispermic origin of XY moles. Nature 292:551-552, 1981.

Panigel M. Anatomy and morphology of the placenta. Clinics in Obstetrics and Gynecology 13:421-445, 1986.

Park W.W. Choriocarcinoma: A study of its pathology. London. Heinemann, 1971.

Pasqualini J.R. and Kincl F.A. Hormones and the fetus. Volume 1. Pergamon Press, 1977.

Patillo R.A. and Gey G.O. The establishment of a cell line of human hormone synthesizing trophoblstic cells *in vitro*. Cancer Res. 28:1231-1236, 1968.

Paul W.E. and Ohara J. B cell stimulatory factor/interleukin-4. Annu. Rev. Immunol. 5:429-460, 1987.

Pijneborg R., Bland J.M., Robertson W.B., Dixon G. and Brosens I. The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy. Placenta 2:303-316, 1981.

Pollard J.W., Arceci R.J., Bartocci A., and Stanley E.R. Colony-stimulating factor-1: a growth factor for trophoblasts? In: Proceeding of Third Banff Conference in Reproductive Immunology, 1989.

Pollard J.W., Bartocci A., Arceci R., Orlofsky A., Ladner M.B. and Stanley E.R. Apparent role for the macrophage growth factor, CSF-1, in placental developement. Nature 330:484-486, 1987.

Psychoyos A. Hormonal control of implantation. Vit. Horm. 31:201-256, 1974.

Queenan J.T., Lao L.Ch., Arboleda C.E., Ulloa Aguirre A., Golos T.G., Cines D.B. and Strauss J.F.III Regulation of urokinase type plasminogen activator production in cultured human cytotrophoblasts. J. Biol. Chem. 262:10903-10906, 1987.

Ramsey E.M. The placenta of laboratory animals and man (J.D. Ebert, ed.) Holt Rinehart and Winston, 1975.

Redman C.W.G. Immunology of the placenta. Clinics in Obstetrics and Gynecology 13:469-499, 1986.

Rennick D., Young G., Gemmell L. and Lee F. Lypopolysaccharide and interleukin-1 inducible production of colony-stimulating factors. Blood 69:682-686, 1987.

Reisbach G., Sinderman J., Kremer J., Hultner L. and Dormer P. Macrophage Colony Stimulating Factor expression in spontaneously outgrown EBV-B cell lines and activated normal B lymphocytes. Blood 74:959-964, 1989.

Rettenmeier C., Sacca R., Furman W.L., Roussel M.F., Holt J.T., Nienhuis A.W., Stanley E.R. and Sherr C.J. Expression of the human c-*fms* oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. J. Clin. Inves. 77:1740-1746, 1986.

Robertson W.B. Uteroplacental vasculature. J. Clin. Pathol. (Supp) 29:10, 1976.

Robertson W.B., Brosensn I. and Dixon G. The ultrastructure of the human placental bed. J. Pathol. 112:203-214, 1974.

Rosal L., Saxena B.B. and Landesman R. Application of the radioreceptor assay for hCG in the diagnosis of early abortion. Fertil. Steril. 26:1105,1975.

Roussel M.F., Downing J., Rettenmeier C.W. and Sherr C.J. A point mutation in the extracellular domain of the human CSF-1 receptor (c-*fms* proto-oncogene) activates its transforming potential. Cell 55:979-988, 1988.

Saxena B.B. Human chorionic gonadotrophin. In: Endocrinology of pregnancy (F. Fuchs and A. Klopper, eds.) Philadelphia: Harper and Row, 1983. pp 50-71.

Saxena B.B. and Landesman R. Diagnosis and management of pregnancy by the radioreceptor assay of hCG. Am. J. Obstet. Gynec. 131:97, 1978.

Scheffler J.T., Fleissner L., Seeling G., Tattanahalli L. and Trotta P. Characterization of the human granulocyte-macrophage receptor from human placenta. J. Cell. Biochem. (Supp) 13C:30, 1989.

Shen S.J., Wang C.Y., Nelson K.K., Jansen M. and Ilan J. Expression of insulin-like growth factor II in human placentas from normal and diabetic pregnancies. Proc. Natl. Acad. Sci. (USA) 83:9179-9182, 1986.

Sherman M. Implantation of mouse blastocysts *in vitro*. In: Methods in mammmalian reproduction. (J.C. Daniel, ed.) New York, Academic Press, 1978.

Sherr C.J., Rettenmeier C.W., Sacca R., Roussel M.F., Look A.T. and Stanley E.R. The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor CSF-1. Cell 41:665-676, 1985.

Silberstein D.S., Owen D.F., Gasson J.C., Di Persio J.F., Golde D.W,, Bina J.C., Soberman R., Austen K.F. and David J.R. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by recombinant granulocyte-macrophage colony-stimulating factor. J. Immunol. 137:3290, 1986.

Smith K.	Interleukin-2: inception, impact and implications. Science 240: 1169-1176, 1988.
Sporn M.B.	and Todaro G.J. Autocrine secretion and malignant transformation of cells. N. Eng. J. Med. 303:771-777, 1980.
Stanley E.R.,	Guilbert L.J., Tushinski R.J. and Bartelmez S.H. CSF-1, a mononuclear phagocyte lineage-specific hemopoietic growth factor. J. Cell. Biochem. 21:151-159, 1983.
Stevens V.C.	Immunization with hormones. In: Reproduction research. Amsterdam: North Holland Publishers, 1975. p 217.
Strickland S.	and Reich E. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-240, 1976.
Thorens B.,	Mermod J.J. and Vassalli P. Phagocytosis and inflamatory stimuli induce GM-CSF mRNA in macrophages through post-transcriptional regulation. Cell 48:671-679, 1987.
Tsai S.,	Emerson S.G., Sieff C.A. and Nathan D.G. Isolation of a human stromal cell strain secreting hemopoietic growth factors. J. Cell. Physiol. 127:137, 1979.
Vaitukaitis J.L.	Changing placental concentrations of hCG and its subunits during gestation. J. Clin. Endoc. Metab. 38:755-760, 1974.
Vaitukaitis J.L.	and Ebersole E.R. Evidence for altered synthesis of human CG in gestational trophoblastic tumors. J. Clin. Endoc. Metab. 42:1048-1052, 1976.
Vaitukaitis J.L.	Glycoprotein hormones and their subunits. Immunological and biological characterization. In: Structure and function of gonadotrophins (K.W. McKerns, ed.) New York: Plenum Press, 1978. p 339.
Varma S.K.L.,	Dawood M.Y., Haour F., Channing C.P. and Saxena B.B. Gonadotrophin-like substance in the preimplanted rabbit blastocyst. Fertil. Steril. 31:68, 1979.

Wake N., Seki T., Fujita H., Okubo F., Sakai K., Okuyama K., Hayashi H., Shiina Y., Sato H., Kuroda M. and Ichinoe K. Malignant potential of homozygous and heterozygous complete moles. Cancer Research 44:1226-1330, 1984.

Wake N., Takagi N. and Sasaki M. Androgenesis a cause of hydatidiform mole. J. Natl. Cancer Inst. 60:51-53, 1978.

Warren M.K. and Ralph P. Macrophage colony-stimulating factor stimulates human monocyte production of interferon, tumor necrosis factor and colony-stimulating activity. J. Immunol. 137:2281-2285, 1988.

Wegmann T.G. Fetal protection against abortion: is it immunosuppresion or immunostimulation? Ann. Immunol (Inst. Pasteur) 136D:309-312, 1984.

Weisbart R.H., Golde D.W., Clark S.C., Wong G.G. and Gasson J.C. Human granulocyte-macrophage colony-stimulting factor is a neutrophil activator. Nature 314:361363, 1985.

Weiss T.J., Janson P.O., Porter K.J. and Seamark R.F. Effects of human chorionic gonadotrophin on the release of cyclic AMP and progesterone from the sheep ovary at different stages of the estrous cycle. Acta Endoc. 89:159-165, 1978.

Wing J.N., Ampel A., Waheed A. and Shadduck R.K. Macrophage colony-stimulating factor enhances the capacity of murine macrophages to secrete oxygen reduction products. J. Immunol. 135:2052-2055, 1985.

Wong G.G., Temple P.A., Leary A.C., Witek-Gianotti J.S., Yang Y.C., Ciarletta A.B., Chung M., Kriz R., Kaufman R.J., Ferenz C.R., Sibley B.S., Truner K.J., Hewick R.M., Clark S.C., Yanie N., Yokota H., Yamad M., Saito M., Motoyoshi K. and Takaku F. Human CSF-1: molecular cloning and expression of a 4 kb cDNA encoding the human urinary protein. Science 235:1504-1508, 1987.

Woolford J., Rothwell V. and Rohrschneider L. Characterization of the human c-*fms* gene product and its expression in cells of the monocyte-macrophage lineage. Mol. Cell Biol. 5:3458-3466, 1985.

Wu M., Cini J. and Yunis A. Purification of a colony-stimulating factor from cultured pancreatic carcinoma cells. J. Biol. Chem. 254:6226-6231, 1979.

Wynn R.M. Immunological implications of comparative placental ultrastructure. In: The Biology of the blastocyst (R.J. Blandau, ed.) Chicago: University of Chicago Press, 1971.

Yagel S., Parhar R.S., Jeffrey J.J. and Lala P.K. Normal nonmetastatic human trophoblast cells share *in vitro* invasive properties of malignant cells. J. Cell. Physiol. 136:455-462, 1988.

Yoshimoto Y., Wolfsen A.R., Hirose F. and Odell W.D. Human chorionic gonadotrophin-like material: presence in normal human tissue. Am. J. Obstet. Gynec. 134:729-734, 1979.

Young D.C., Wagner K. and Griffin J.D. Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. J. Clin. Invest. 79:100-104, 1987.

Zeilmaker G.H. and Verhamme CM. Luteotropic activity of ectopically derived rat blastocysts. Acta Endocrinologica 88:589, 1978.

CHAPTER 2 FIRST SEPARATE STUDY:

HEMOPOIETIC COLONY-STIMULATING FACTORS INDUCE THE DIFFERENTIATION OF NORMAL HUMAN TROPHOBLASTS.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage CSF (CSF-1) are lympho-hematopoietic cytokines originally characterized in part for their role in mononuclear phagocyte differentiation, proliferation and function (1). Both cytokines have been recently implicated in placental development: uterine CSF-1 levels increase over 10,000 fold during the murine pregnancy and mRNA to the CSF-1 receptor (c-*fms*) has been localized to the giant cell layer of the murine trophoblast (2). GM-CSF has been found at the murine trophoblast-decidua interface (3) and its receptor has been cloned from a placental cDNA library (4). Although both CSFs appear able to stimulate placental cell growth in mouse (3,5), responsive cells within the human placenta have not been identified and the two cytokines' function, especially in human, remains largely undefined. We here show that both GM-CSF and CSF-1 target the major cell population in the human placenta, trophoblasts, and that both stimulate differentiation of cytotrophoblasts to syncytium. In addition both cytokines stimulate the production in culture of the pregnancy related hormones placental lactogen (hPL) and chorionic gonadotropin (hCG). These data show that the same cytokines that support hematopoietic phagocyte (macrophage) growth, differentiation and function have very similar functions on placental epithelial phagocytes (trophoblasts).

In order to determine which cells respond to CSF-1, we first localized expression of c-*fms* mRNA, which encodes the CSF-1 receptor (6), to a placental cell type. We have previously reported that a preparation of villous cytotrophoblasts, strongly expressed c-*fms* mRNA (3). In order to determine whether active CSF-1 receptor protein was expressed, these cells were incubated for 18 hours to upregulate receptors to the cell surface then assessed for ability to bind ^{125}I-CSF-1 at 4°C. As in mouse macrophages (7), specific high affinity binding of ^{125}I-CSF-1 to isolated human term trophoblasts is irreversible at 4°C (data not shown). Therefore receptor binding is saturable when the concentration of ligand is greater than receptor and is high enough to allow rapid occupation of the somewhat labile receptor (e.g. ref 7). Under these conditions (1.4 nM ^{125}I-CSF-1), saturation binding was observed after 1 hour of incubation on ice and corresponded to approximately 1600 receptors per cell (Table 2.1).

Microscopic examination revealed that during the 18 hour upregulation period, a substantial proportion of cytotrophoblasts coalesce to form cellular aggregates, some of which appeared to have fused into multinuclear syncytial structures (average of 5 nuclei per structure), a phenomenon known to occur spontaneously in serum containing cultures (8). Autoradiography of trophoblast preparations after ^{125}I-CSF-1 binding showed that 66% of the cellular structures present in the culture expressed CSF-1 receptors (Figure 2.2). Occasionally heavily labeled single cells were observed but the highest densities of cell-associated grains were present on multinucleated structures suggesting that the levels of expression of CSF-1 receptor protein increases as cytotrophoblasts differentiate in syncytial elements. This is in agreement with observations made by *in situ* hybridization that c-*fms* mRNA is present at highest density in the syncytium of both first trimester and term placental villi (Figure 2.1). We next determined whether the CSF-1 receptor could mediate any function on isolated trophoblasts.

The rate of trophoblast proliferation is maximal during the first weeks of pregnancy, declining thereafter, although cytotrophoblasts proliferate and form syncytium until term (9). We examined the ability of CSF-1 (and GM-CSF) to stimulate DNA synthesis over a 72 hour culture period. However, we were unable to detect any significant ^{3}HTdR uptake under any culture conditions after the first 12 hours of incubation and thus are unable to make any conclusions regarding growth stimulation (data not shown).

The trophoblast differentiates by the fusion of cytotrophoblast cells to form a syncytium (10), which is the main producer of human chorionic gonadotropin (hCG) and plancental lactogen (hPL). HCG is responsible for the maintenance of corpus lutem function in early pregnancy (10), and hPL is responsible for a variety of metabolic changes that occur during pregnancy, as well as being an indirect regulator of fetal growth (11). We therefore wished to determine whether CSF-1 and GM-CSF had effects on both differentiation and hormone secretion.

CSF-1 stimulated the spontaneous syncytialisation of cytotrophoblast in culture (Fig 2.3.b). In 8 separate experiments, CSF-1 also stimulated a significant rise in hCG secretion (1.71 ± 0.15 fold, $p<0.05$) for the first 5 days after CSF-1 addition (Figure 2.4.a). A smaller, but significant, rise in hPL secretion was observed for the first 4 days following CSF-1 addition to the cultures (1.35 ± 0.10, $p<0.05$). In comparison, GM-CSF induced a striking morphologic differentiation of cytotrophoblast into syncytium (Fig 2.3.c). Control cultures showed only some aggregation and small syncytial formation, but GM-CSF markedly increased not only the extent but the rate of syncytium formation, so that within 96 hours only large sheets of multinucleated syncytial structures were observed. In some cultures there appeared to be a qualitatively greater amount of differentiation than that seen with CSF-1. GM-CSF induced a different pattern of hormone secretion (Fig 2.4.b). In six separate experiments, 100 U/mL of GM-CSF induced a large and prolonged release of hPL over 5 days of culture (2.4 ± 0.30, $p<0.005$), but only a small transient increase in hCG for the first three days of culture (1.22 ± 0.07, $p<0.01$). These changes in hormone secretion are not due to cell proliferation, as we could demonstrate neither increased cell number nor ^{3}HTdR uptake (data not shown).

These data demonstrate the expression of specific high affinity CSF-1 receptors on the surface of cytotrophoblast cells and that show these receptors can mediate the CSF-1 stimulated induction of cytotrophoblast differentiation and subsequent increase in hCG and hPL secretion. Receptors for GM-CSF have been demonstrated in placental membranes (4) and in a trophoblast cell line (15). In addition, we have recently shown that GM-CSF acts as an autocrine regulator for choriocarcinoma cell lines derived from transformed trophoblast (12). In the current experiments, GM-CSF markedly induced differentiation, in some cultures more so than observed with CSF-1. Interestingly, neutralizing antiserum to GM-CSF tended to reduce the spontaneous differentiation to syncytium in culture, indicating the latter phenomenon to be due to endogenous production of GM-CSF in cultures of purified cytotrophoblast. In addition, there was a clear preponderance of hPL secretion compared to hCG. We have previously noted that EGF induces cytotrophoblast differentiation and secretion of hCG and hPL in parallel, implying that the increases in hormone production were linked to differentiation. In contrast, GM-CSF induced secretion of hPL and hCG to differing degrees. These finding suggest that although secretion of hPL and hCG may be a function of the differentiated state, individual cytokines, and GM-CSF in particular, may have specific effects on the expression and secretion of individual hormones.

Our data show that in the human, as in the mouse, the lympho-hematopoietic cytokines CSF-1 and GM-CSF have important functions in pregnancy in the development and function of placental trophoblast, a tissue of epithelial, and distinctly non-lympho-hematopoietic, origin. This point reveals two interesting considerations. The first is that these observations bring trophoblast development and function within the regulatory network encompassing the immune system and therefore support the idea that the maternal immune response to the developing conceptus can be beneficial (13). These data also indicate a parallel development of hematopoietic and placental phagocytes both in terms of overlaping properties and functions (expression of Fc receptors, phagocytosis, expression of CD4 antigen and ability to form either extended [trophoblast] or limited (tissue giant cells) syncytium] but also in regulatory responsiveness to two classic macrophage growth factors CSF-1 and GM-CSF.

Figure 2.1 Localization of c-*fms* mRNA in first trimester human placental villi by *in situ* hybridization.

Tissues obtained from elective termination of pregnancy were washed extensively in RNAse free phosphate buffer saline, immediately fixed in 4% paraformaldehyde and processed as described by Miller *et al* (14). A c-*fms* specific RNA probe (500 base pairs) was generated form the full length human c-*fms* cDNA by an *in vitro* transcription system according to the manufacturer instructions (Omega) and labeled with ^{35}S. Hybridization was performed for 18 hours at 53°C, followed by RNAse treatment and high stringency washings. Slides were then dried, immersed in Kodak photographic emulsion and exposed for 14 days. Negative controls included a) pretreatment of the tissues with excess RNAse and b) hybridization with a non-specific probe (specific for murine class I antigen) (not shown).

The highest density of grains (corresponding to the sites of maximal hybridization) is observed in the periphery of the chorionic villi in a transversal section.

Figure 2.1 Localization of c-*fms* mRNA in first trimester human placental villi by *in situ* hybridization.

Figure 2.2 Expression of high affinity binding sites for ^{125}I-CSF-1 on cultured human term trophoblasts.

Cytotrophoblasts were prepared by eight sequential 10 minute digestions of normal human term placental tissues using 0.25% trypsin and 10 μl/ml of DNAse type I as described by Morrish *et al* (8). Cells were cultured on tissue culture treated chamber slides in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 5% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. The purity of the isolated population of cells was assessed by immunostaining with anti-cytokeratin (specific for epithelial cells) and anti-vimentin antibodies (for cells of mesenchymal origin) as well as with anti-HCG antibodies (considered a trophoblast marker) and was always greater than 90%. After 18 hours of incubation (to allow for adherence and receptor upregulation), the cells were rapidly cooled to 4°C on ice and ^{125}I human rCSF-1 [labeled to 3 x 10^8 cpm/mol as described previously (7), and used at approximately 300 pM] was added. Binding was carried out in IMB-10% FBS (IMDM without sodium bicarbonate, brought to pH 7.4 with sodium hydroxide) at 2°C for a total of 2 hours. Non-specific binding was determined as previously (7) by blocking high affinity binding sites with unlabeled CSF-1 (2 hour pre-incubation at 4°C, see ref 7). After the binding reaction, cells were washed extensively with cold PBS, fixed on the slides with methanol, coated with Kodak NTP2 emulsion (diluted 1:1 in water), dried and exposed for 14 days before development.

The black grains above the multinucleated syncytial structure (centre) correspond to the specific binding sites of ^{125}I labeled CSF-1.

Figure 2.2 Expression of high affinity binding sites for ^{125}I-CSF-1 on cultured human term trophoblasts.

Figure 2.3 Effect of CSF-1 and GM-CSF on trophoblast morphological differentiation.

Trophoblasts were isolated and characterized as descescribed in the legend to Fig 1.2. Cells (2×10^5/ml; 3 ml total volume) were seeded in 35 mm tissue culture plates and cultured in IMDM-5% FBS for 4 days. Cultures were then washed gently with warm medium, fixed in methanol and stained with May-Grumwald/Giemsa.

2.3. a) Control cultures (medium alone)

2.3. b) Cells cultured in the presence of 1000 u/ml of human recombinant CSF-1.

2.4. c) Cells cultured in the presence of 100 u/ml of human recombinant GM-CSF.

Figure 2.3 Effect of CSF-1 and GM-CSF on trophoblast morphological differentiation.

a) Control

Figure 2.3 Effect of CSF-1 and GM-CSF on trophoblast morphological differentiation.

b) CSF-1

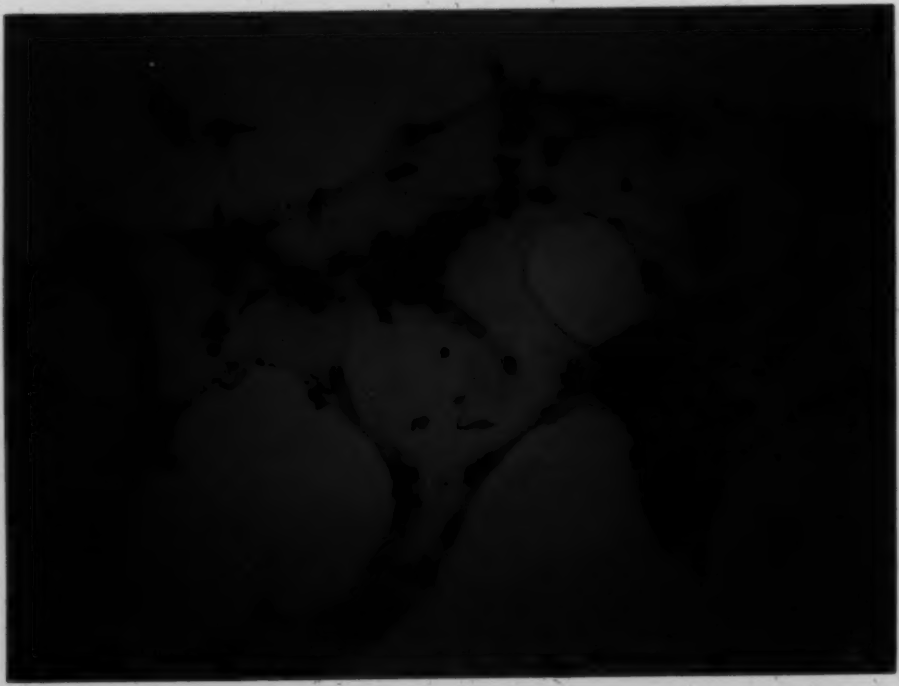

Figure 2.3 Effect of CSF-1 and GM-CSF on trophoblast morphological differentiation.

c) GM-CSF

Figure 2.4 Effect of CSF-1 and GM-CSF on hCG and hPL production by human term trophoblasts.

Cytotrophoblasts were prepared as described in the legend to Fig 2.2, plated in 24 well tissue culture dishes (Corning) in IMDM-10% FBS to allow cell attachment. After 4 hours, medium was changed to serum free IMDM, which was changed daily and kept frozen for hormone determinations. Human recombinant CSF-1 (5000 μ/ml) or human recombinant GM-CSF (100 μ/ml) in 0.01% bovine serum albumin (BSA) in PBS (20 μl/culture) or control BSA/PBS was added daily after each medium change. HCG and hPL were assayed by a specific radioimmunoassay as previously described (8).

2.4. a) Effect of CSF-1 on hCG and hPL production. The results of 8 separate experiments are displayed. Ordinate: mean ± SD of the fold increase relative to control cultures. Abcissa: culture time in days.

2.4. b) Effect of GM-CSF on hCG and hPL release by trophoblasts in culture. The results of 6 separate experiments are displayed.

Figure 2.4 Effect of CSF-1 and GM-CSF on hCG and hPL production by human term trophoblasts.

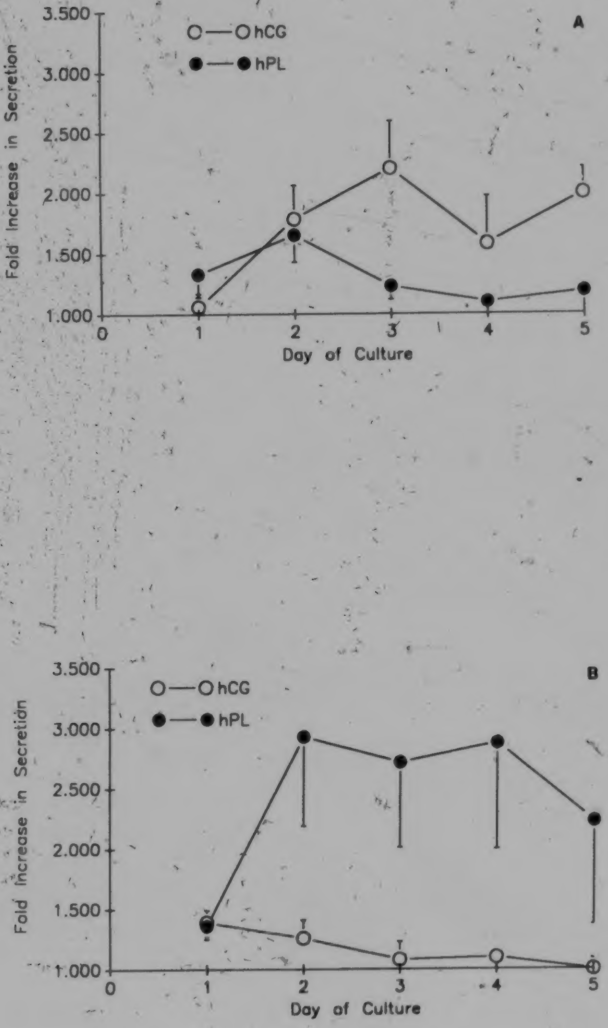

Table 2.1 Binding of ^{125}I-CSF-1 to Normal Human Trophoblasts as a Function of Time at 4° C*

Incubation Time (min)	Net Molecules/cell bound ± SD
30	743 ± 44
60	763 ± 21
120	1595 ± 97
240	1678 ± 262

* Concentration ^{125}I-CSF-1 = 1.4 x 1^{-9} M

Specific Activity = 4 x 10^{18} cpm/mol

BIBLIOGRAPHY

1. Stanley ER, Guilbert LJ, Tushinski RJ, Bartlemez SH (1984) In: Mononuclear Phagocyte Biology (Volkman A, Ed) M Dekker, Inc, New York, Basel, p 373.

2. Regenstreif LJ, Rossant J. (1989) Devel. Biol. 133: 284.

3. Guilbert LJ, Athanassakis I, Branch DR, Christopherson R, Crainie M, Garcia-Lloret M, Mogil RJ, Morrish D, Ramsoondar J, Vassiliadis S, Wegmann TG (in press) In: The Molecular and Cellular Immunobiology of the Maternal Fetal Interface. (Gill TG, Wegmann TW, Eds).

4. Gearing DP, King JA, Gough NM, Nicola NA (1989) EMBO J 8: 3667.

5. Athanassakis I, Bleackley RC, Paetkau V, Guilbert LJ, Barr PJ, Wegmann TG (1987) J Immunol 138: 37.

6. Sherr CJ, Rettenmeier CW, Sacca R, Roussel MF, Look AT, Stanley ER (1985) Cell 41: 665.

7. Stanley ER, Guilbert LJ (1981) J Immunol. Meth. 42: 253.

8. Morrish DW, Bhardwaj D, Dabbagh LK, Marusyk, Siy O (1987) J Clin Endocrin Metab 65: 1282.

9. Pfeiffer-Ohlsson S, Goustin AS, Rydnert J, Wahlstrom T, Bjersing, Stehelin D, Ohlsson R (1984) Cell 38: 585.

10. Tulchinsky D, Ryan KJ (1980) Maternal-Fetal Endocrinology, WB Saunders Co., Phil.

11. Talamantes F, Ogren L, Markoff E, Woodard S, Madrid J (1980) Fed Proc 39: 2582.

12. Garcia-Lloret M, Turner AR, Honore L, Wegmann TG, Guilbert LJ (submitted)

13. Wegmann TG (1984) Ann Immunol (Paris) 135D: 301.

14. Miller FD (1987) J Cell Biol 105:3065.

15. Di Pesio JF, Golde DW, Gasson CA (1990) Int J Cell Cloning 8(Supp1): 63.

CHAPTER 3 SECOND SEPARATE STUDY:

CHORIOCARCINOMA CELLS ARE AUTOCRINE FOR TWO HEMATOPOIETIC GROWTH FACTORS

INTRODUCTION

The hematopoietic cytokines CSF-1, GM-CSF and IL-3 were originally identified by their ability to stimulate the proliferation, differentiation and survival of hematopoietic cells *in vitro* (1). While CSF-1 specifically promotes the production of mononuclear phagocytes (2) GM-CSF and IL-3 are pluripoietins by virtue of their capacity to stimulate progenitors of several hematopoietic lineages. The cytokines have been well characterized and have been molecularly cloned (3 ,4 ,5) as have been their receptors (or receptor subunits (6, 7, 8).

Recently, novel functions for hematopoietic cytokines outside the hematopoietic system have been described. Functional GM-CSF receptors have been identified in a variety of cell lines of human origin: small cell lung and breast carcinoma, osteosarcoma, bone marrow stromal fibroblasts (9, 10) as well as in choriocarcinomas and normal placental membranes (11) from whence the receptor was cloned (7). In many instances, a proliferative response to GM-CSF was observed. Expression of the transcripts of the proto-oncogene c-*fms*, which encodes the CSF-1 receptor, has also been demonstrated in choriocarcinoma cell lines and in placental tissues (12). Interestingly, CSF-1 levels increase 10,000-fold in mouse uterus during pregnancy (13): *in situ* hybridization demonstrates the preferential localization of anti-sense mRNA to the luminal and glandular epithelium of the endometrium in mouse (14). Furthermore, murine placental cells of fetal origin with the characteristics of both macrophages and trophoblasts specifically bind CSF-1 and proliferate in response to CSF-1, GM-CSF and IL-3 (15).

The abnormalities of malignant human trophoblast growth manifest a wide range of biological behaviors. Three distinct clinico-pathological entities are recognized: hydatidiform mole, invasive moles and choriocarcinomas (16). Fifty percent of choriocarcinomas arise from untreated moles (17). While the mole is self-limiting by nature, choriocarcinomas are highly metastatic and invasive tumors. Little is known about the factors involved in this progression.

The autocrine hypothesis proposes that a cell produces hormone-like substances that can interact with specific membrane receptors on its surface to induce effects such as proliferation (18). Aberrant synthesis of growth factors may lead to autonomous growth and malignant transformation in cells that express the corresponding receptor (19, 20). In this work we have asked whether autocrine stimulation by macrophage growth factors (MGFs) could be involved in choriocarcinoma cell growth. To investigate this question, we have studied the effects of pure recombinant CSF-1 and GM-CSF on three well characterized human choriocarcinoma cell lines (BeWo, JEG-3, JAR) and have measured the production of these factors by the cells *in vitro*. We show that the three cell lines constitutively express high affinity receptors for CSF-1 and respond mitogenically to it, whereas no response was obtained under similar conditions with GM-CSF. Synthesis of biologically active CSF-1 and GM-CSF was detected in the three cell lines. To determine whether extracellular interactions of secreted factors with cell surface receptors might lead to unregulated autocrine growth, we investigated the effect of neutralizing antibodies to GM-CSF and to the CSF-1 receptor on the proliferation of the cells and found both antibodies able to inhibit the autonomous growth of the three cell lines by more than 50%.

MATERIALS AND METHODS

Cell Lines

The human choriocarcinoma cell lines BeWo, JAR and JEG-3 (American Type Culture Collection) were grown in Iscove's Modified Dulbecco medium (IMDM, Gibco, Grand Island, N.Y.) supplemented with 10% (v/v) fetal bovine serum (FBS Flow Laboratories, Mac Lean , VA) at 37° C in 5% CO_2.

Colony Stimulating Factors

Recombinant human CSF-1 was obtained as the protein purified from medium conditioned by CHO cells transfected with the 'short' form of CSF-1 cDNA (3) and was obtained through Dr. P. Ralph (Cetus Corporation, Emeryville CA). IL-3 was used as a high potency supernatant derived from COS 7 cells transfected with the pXM expression plasmid and was obtained through Dr. S. Clark (Genetics Institute). Recombinant human GM-CSF was a gift from Dr. P Mannoni (INSERM, Marseille, France).

I CSF-1 Binding

Pure recombinant human CSF-1 was radio-iodinated with the chloramine T method as previously described to approximately 4 x 10^{18} cpm/mole (46). The biological activity of each iodinated preparation was determined using a previously described colorimetric method (47). The labeled factor was used within two weeks of iodination provided its biological activity was >80%. Binding of ^{125}I-CSF-1 was performed at 2° C in 96 well flat bottomed microtiter plates as previously described (47). Log phase growing choriocarcinoma cells were harvested by trypsinization, washed three times in phosphate buffered saline (PBS), resuspended in IMDM-FBS and aliquoted at 1×10^5 cells per well per 100 ul. After overnight incubation to allow for complete attachment, plates were washed twice with ice cold PBS and 50 ul of IMB (IMDM lacking bicarbonate adjusted to pH 7.35 with NaOH) containing 10% FBS was added to each well and the plate cooled on ice for 15 minutes. Iodinated CSF-1 (5×10^5 cpm / 20ul / well = 2.4×10^{-9} M) was then added and the binding reaction carried out on ice for 2 hours, after which the wells were carefully washed five times with ice cold PBS. Cell associated ^{125}I was recovered from the plates solubilizing the contents of each well with 100 ul of 0.5% SDS in 0.1 M Tris and the radioactivity was quantitated in a gamma counter. Preliminary experiments showed that binding to choriocarcinoma, cell lines like that to murine macrophages (21), was irreversible at 4° C and complete at 2 hours at ^{125}I-CSF-1 concentrations > 10^{-9}M. Under these conditions specific binding, defined as the binding that can be inhibited by a pre-incubation with 2 nM unlabeled CSF-1 (21) reached plateau levels at 1 nM ^{125}I-CSF-1. The number of high affinity binding sites per cell was calculated using the formula:

$$\text{sites/cell} = \frac{\text{specific binding (cpm/well)} \times 6.02 \times 10^{23} \text{ molecules}}{4 \times 10^{18} \text{ cpm/mol} \times 10^{5} \text{ cells/well}}$$

CSF-1 Assay

The content of CSF-1 in supernatants and subcellular preparations was determined with a specific radioreceptor assay (RRA) using the CSF-1 receptor bearing murine macrophage cell line P388D1 (a gift form Dr. K.C. Lee, U of Alberta, Edmonton) as target and ^{125}I human CSF-1 as tracer. The assay was performed in 96 well microtiter plates, on ice. Samples and standards (50 ul/well) were added in triplicate. Control wells include diluent alone (to determine maximum label bound) or 2 nM unlabeled CSF-1 (to determine non-specific binding). A total of 1.5 x 10^5 cells in 50 ul (previously washed three times with ice cold PBS and resuspended in IMB-FCS) were added to each well and incubated for two hours prior to the addition of ^{125}I-CSF-1 (5 x 10^4 cpm/well in 25 ul). After a further 1 hour incubation, 100 ul aliquots from each well were layered on top of 200 ul ice cold FBS in 500 ul polyethylene microfuge tubes. The tubes were immediately centrifuged (10000 rpm, 2 minutes) and rapidly frozen in liquid nitrogen. The ^{125}I content of the tips of the tubes containing the cell pellet (cell count), and the remainder of the tube (free) was determined in a gamma counter. A standard L cell conditioned medium preparation (a gift form Dr. E.R. Stanley) calibrated against a stable standard human urinary CSF-1 preparation was used to obtain a standard curve in each determination.

Cellular Fractionization of CSF-1 Content

The CSF-1 content was determined in four different fractions obtained from the three choriocarcinoma cell lines: a) spent supernatant fluid after 48 hour incubation (fraction I); b) receptor associated CSF-1 :after collecting the supernatant fluid, cells were washed three times with ice cold PBS then 2 mL of cold dissociation buffer (0.1M acetic acid in 100 mM NaCL supplemented with 0.1% FBS and adjusted to pH 4) was added to confluent cultures in 100 mm tissue culture plates and incubated for 5 minutes on ice. The fluid was collected, immediately neutralized with 1 M Tris and twice dialyzed against PBS and finally against IMDM (fraction II); c) after isolating the receptor bound CSF-1, plates were washed twice with PBS and then treated with 1.8 mL 0.1% trypsin in Hank's Balanced salt solution at 37° C to obtain the membrane associated CSF-1. After three minutes the reaction was stopped with 0.2 mL FBS and the 2 mL supernatant saved (fraction III); d) finally, cells were scrapped from the plates in 2 mL 10% FBS in IMDM with a rubber policeman and subjected to 5 cycles of rapid freeze-thaw to release cytoplasmic CSF-1 (fraction IV).

GM-CSF Assay

The amount of GM-CSF secreted by the choriocarcinoma cell lines was quantitated by the proliferation assay described by Griffin *et al.* (49) with some modifications. Briefly, frozen stocks of Ficoll-Hypaque separated mononuclear cells from chronic myelogenous leukemia patients were thawed and cultured for 48 hours in Opti-MEM medium supplemented with 5% FBS and 10% conditioned medium form the human pancreatic carcinoma cell line 5637 as a source of colony-stimulating activity . Cells were then washed three times in PBS and resuspended at 1 x 10^6/mL in Opti-MEM-FBS. Assays were performed in 96 well flat bottomed tissue culture plates. Fifty μL of cells (5 x

10^4) were cultured with 25 μL of sample to be tested in triplicate with or without the addition of 25 μL of a 1:200 dilution (final) of a specific bovine anti-human GM-CSF anti-serum, for 48 hours at 37° C in 5% CO_2. The antibody treatment is necessary to specify the assay since other CSFs are also able to stimulate the proliferation of these cells (e.g. IL-3, 50). Induction of proliferation by CSFs was assessed by ^{3}HTdr uptake. Cells were pulsed for 4 hours with 1 uCi/well of ^{3}HTdr and then harvested onto absorbent glass filter paper and counted in a scintillation counter. Results are expressed in GM-CSF units/mL calculated from alignment of antibody inhibitable cpm to a standard curve prepared with a reference human recombinant GM-CSF preparation.

Clonogenic Assay

Choriocarcinoma cell lines in log phase growth were harvested by trypsinization, washed three times in PBS and plated at 10 cells per well in 100 ul of IMDM supplemented with 10% FBS in six replicate wells in 96 well flat bottomed tissue culture plates with or without different growth factor preparations. At the end of a 48 hour incubation, plates were fixed in methanol and stained with Giemsa. The number of clones (defined as clusters of 8 cells or more) per well was counted using an inverted microscope. Results were expressed as total number of clones/six wells/variable.

Inhibition of Choriocarcinoma Cell Proliferation by Anti-GM-CSF and anti-human CSF-1 Receptor Antibodies.

2×10^2 CC cells were cultured in 100 ul of IMDM supplemented with 2% FBS in the presence or absence of antibodies (1:200 dilution anti-human GM-CSF bovine anti-serum; 1 ug/mL rat anti-human CSF-1 receptor monoclonal antibody, a gift from Dr. C.J. Scherr). Controls included identical concentrations of normal bovine serum and isotype matched rat anti-mouse H-2 monoclonal antibody. Effects of antibodies on proliferation was assessed by ^{3}HTdr uptake after a 48 hour incubation. Cells were pulsed for 4 hours with 1 uCi ^{3}HTdr, harvested and counted in a scintillation counter.

Northern Blot Analysis

Total RNA from choriocarcinoma cell lines or purified trophoblasts isolated from normal term placentas as described (51) was prepared by acid guanidium thiocyanate-phenol-chloroform extraction by a procedure modified form Chomczynski and Sacchi (52). Fifteen ug of total RNA were run on 1% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose paper. The filter was hybridized with a 2.8 kB human c-*fms* cDNA probe labeled with 32P by random oligonucleotide priming (53) for 19 hours at 40 C. Filters were then dried and exposed onto X-ray film for 48 hours.

Statistical Methods

The results were analyzed in apaired t-test with the significance determined by a rndomization test (54). Computations were performed in an IBM PC running RANTEST, a general program for computing rnadomization tests (55).

RESULTS

Choriocarcinomas Actively Transcribe the c-*fms* Proto-oncogene

The CSF-1 receptor is an integral transmembrane glycoprotein with associated tyrosine kinase activity encoded by the c-*fms* proto-oncogene (22). Previous reports have shown the presence of c-*fms* transcripts in the BeWo cell line (23). In an attempt to determine whether this phenomenon is a common feature of all malignant trophoblasts, we looked for the expression of CSF-1 receptor mRNA in the three choriocarcinoma cell lines. As seen in Figure 3.1, all cell lines expressed a 4 kb c-*fms* mRNA species and a similar band can be detected on purified normal trophoblasts . Whether this identity also reflects complete homology at the genomic level remains to be explored, but from our results it can be inferred that expression of c-*fms* message is an intrinsic characteristic of normal and malignant trophoblasts.

Choriocarcinoma Cell Lines Constitutively Express Functional Receptors for CSF-1

The expression of functional CSF-1 receptors on BeWo, JEG-3 and JAR was assessed by their ability to specifically bind ^{125}I-CSF-1 at high affinity. The binding of human ^{125}I-CSF-1 to human choriocarcinoma cell lines at 4° C, like that of the murine cytokine to murine macrophages (21), was irreversible (and therefore could be blocked by pretreatment with 1 nM unlabeled CSF-1) , saturable and was complete by 2 hrs at a concentration of > 1 nM (data not shown). As shown in Table 3.1 the three cell lines exhibit a similar number of unoccupied binding sites (2,500 molecules per cell).

Choriocarcinomas can respond to exogenous CSF-1

These results indicated that choriocarcinomas are able to translate the c-*fms* message into protein expressed as extracellular ligand-binding site. Whether these receptor were functionally able to mediate any detectable mitogenic response remained to be determined.

All three cell lines grow autonomously *in vitro*, even at low serum concentrations. Attempts to determine a proliferative response to exogenous cytokines by ^{3}HTdr incorporation at relatively high cell densities (0.5 to 5 x 10^4 cells/mL) were unsuccessful (data not shown). When cells were plated at low densities (2 x 10^2/mL), they grew poorly and most only completed one or two cell divisions after 72 hours, suggesting absence of a self supplied growth requirement. Addition of 3000 U/mL of human recombinant CSF-1 consistently increased the number of clonogenic cells in the culture by 1.5 to 2 fold (Table 3.2). This modest, but highly significant ($p < 0.001$) stimulation, was reproducibly observed in 9 individual experiments. Il-3, another myeloid growth factor shown to stimulate the proliferation of murine placental cells (15) also improved the clonogenic growth of JAR and JEG-3 but it had no effect on the BeWo cell line. In contrast, GM-CSF, a cytokine also implicated in murine placental cell development did not stimulate the clonal growth of any of the cell lines (data not shown).

JAR, BeWo and JEG-3 Secrete CSF-1 *in vitro*

The demonstration of the synthesis of a growth factor by a cell that expresses its receptors constitutes a *sine qua non* condition in the formulation of an autocrine loop. Virally transformed human placental cells have been reported to express high levels of CSF-1 mRNA (3). Unknown is whether normal or malignant trophoblasts are able to synthesize the biologically active factor. Using a specific radioreceptor assay we quantitated the levels of CSF-1 in cell supernatants as well as in subcellular extracts (Table 3.3) Choriocarcinomas constitutively secreted CSF-1 into the culture medium. That part of the secreted molecules rebind to the cell surface receptor was demonstrated by the observation that a considerable amount of CSF-1 can be recovered after treatment of the cells at low pH, a condition known to favor the dissociation of the ligand from its receptor (24). Since the assay employed only detects biologically active CSF-1 as judged by its ability to compete with radiolabeled factor for the binding on a CSF-1 receptor expressing murine macrophage cell line, we conclude that choriocarcinomas are able to synthesize a functional molecule with receptor binding properties similar to native human CSF-1. In addition, we found that the secreted material is bioactive by criteria of its ability to stimulate the proliferation of a CSF-1 dependent macrophage cell line (5/10.14, ref 41) (data not shown).

Anti CSF-1 Receptor Antibodies Inhibit the Growth of Choriocarcinoma Cell Lines

The above observations strongly suggested the presence of a CSF-1 driven autostimulatory mechanism underlying choriocarcinoma cell growth. To further strengthen our hypothesis, we asked whether anti-CSF-1 receptor antibodies could inhibit the spontaneous growth of these cells *in vitro*. Since the three lines expressed similar levels of CSF-1 and its receptor, ability of anti-CSF-1 receptor to block growth was tested exclusively on BeWo. Monoclonal anti-CSF-1 receptor antibody was able to inhibit the growth of this cell line by 85% (Table 3.4). A moderate inhibitory activity (25%) was always observed with different rat IgG_{2b} antibodies used as controls. These results indicate that the interaction of secreted CSF-1 with its cognate receptor expressed at the cell surface is at least partially involved in the autonomous growth of choriocarcinoma cell lines.

Evidence for an Additional Autocrine Loop Involving Secretion of GM-CSF

GM-CSF has been implicated as a growth factor for a variety of malignancies of non-hemopoietic origin (9,10). The recent observation that the JAR cell line expresses GM-CSF receptors(11) prompted us to investigate whether the autocrine secretion of this cytokine could be involved in the autonomous growth of choriocarcinoma cell lines.

Synthesis of GM-CSF by JAR, JEG-3 and BeWo was assessed by means of a CML proliferation assay specific by anti-GM-CSF antibody (see methods). All cell lines secreted a CML blast stimulatory activity that could be partly blocked by anti-GM-CSF anti-serum (Table 3.5). Treatment with antibody had no effect on the IL-3 or G-CSF driven proliferation of CML blasts (Table 3.6) 6), further confirming that the detected activity was indeed GM-CSF.

To determine whether secreted GM-CSF stimulated the cell growth of choriocarcinoma cells, the effects of anti-GM-CSF antibodies on autonomous proliferation was tested. The incorporation of ^{3}HTdr into cellular DNA in calf anti-GM-CSF antibody treated and control cultures was measured. Anti-GM-CSF antibody inhibited the proliferation the three cell lines by an average of 40% (range 25% to 65% in nine independent experiments) . Results of a repreentative experiment are shown in Table 3.7 . This inhibition could be reversed by preincubating the anti-serum with an excess of GM-CSF indicating that the effect observed was specific . These findings strongly indicate that a portion of the autonomous growth of choriocarcinoma cell lines can be attributed to response to autocrine secreted GM-CSF. The observation that anti-GM-CSF antibody could not completely inhibit the autonomous growth of these cells implies that additional mechanisms are involved in this process and is in accordance with our observations that CSF-1 might also be involved (see above).

DISCUSSION

The production of growth factors by transformed cells has led to the suggestion that autocrine growth stimulation may be of importance for the maintenance of the transformed phenotype (25). Hemopoietic growth factors have been implicated as autocrine growth stimulators in a variety of hemopoietic malignancies (26-29). In this study have presented evidence that GM-CSF and CSF-1, two cytokines primarily involved in the proliferation and differentiation of myeloid cells, may also act as autocrine stimulators in tumors of non-hematopoietic origin, namely in trophoblastic malignancies. Our data show that choriocarcinoma cell lines express functional CSF-1 receptors and are able to synthesize CSF-1 and GM-CSF in culture. That the autocrine secretion of these factors is causally associated with the autonomous proliferation of choriocarcinomas *in vitro* is further demonstrated by the observation that neutralizing antibodies to the CSF-1 receptor or GM-CSF significantly inhibit the growth of the three cell lines studied. Since permanent cell lines may have additional genotypic alterations as a consequence of establishment, the evaluation of the role of CSF-1 and GM-CSF in the *in vivo* neoplastic transformation will be critically dependent on studies on primary tissues.

It is generally thought that malignant tumors arise through a complex multistep process whereby a cancer cell acquires the capacity for extended proliferation, invasion and metastasis as a result of specific mutations or deregulation of normal genes (30). The loss of exogenous growth factor dependence that leads to the apparent autonomous proliferation of tumor cells is considered to be an important stage in this spectrum (31). *In vitro* studies have shown that some factor dependent cell lines concomitantly acquire the capacity to produce self-stimulatory growth factors and to become tumorigenic (32), suggesting a direct link between autocrine stimulation and malignant transformation. Similar observations have been made in genetically engineered autocrine systems (33,34).

While not all cells rely on autocrine mechanisms for evolution to the malignant phenotype (35), it is also true that the potential for autocrine stimulation is not unique to tumor cells. Macrophages, T cells and endothelial cells are able to produce several growth factors to which they are known to respond (36-38) and there is some evidence that the growth of normal trophoblasts may in part be regulated in an autocrine manner by platelet derived growth factor (PDGF) (39) and chorionic gonadotrophin (HCG) (40). Autocrine regulatory mechanisms may be in fact a common feature of conditions that require extraordinary functional and proliferative demands such as embryogenesis, infection and tissue repair.

Synthesis of self-stimulatory molecules in normal cells appears to occur mainly after induction by exogenous stimuli, for a limited period of time and is tightly regulated at the transcriptional and post-transcriptional level (41, 42) whereas tumor cells produce growth factors constitutively. Self-stimulated uncontrolled growth could therefore be caused by quantitative differences in growth factor production due to a failure in the normal regulatory mechanisms that restrain their synthesis in normal cells. Qualitative differences can also arise when genetic alterations lead to the aberrant expression of a normally silent growth factor gene in cells that express the respective receptor. Abnormalities in the site of interaction or in the intracellular degradative pathways followed by autocrine growth factors and their receptors have also been implicated as mechanisms in growth factor mediated malignant transformation (43). Normal human trophoblasts express CSF-1 and GM-CSF receptors and respond to these factors by morphological differentiation and increased hormone production (44) but it is unknown whether these cells are able to synthesize CSF-1 and GM-CSF. Therefore, from our data we cannot presently distinguish between above mechanisms of abnormal autocrine stimulation.

Choriocarcinomas are remarkably similar to normal trophoblasts both at the structural and the biochemical level and, in contrast with many other tumors, retain some functional capacities characteristic of the normal differentiated tissue (16). Normal trophoblasts, in turn resemble tumor cells in many aspects, they proliferate at high rate in an apparently autonomous manner, invade local tissues and are able to spread through the bloodstream and metastasize in distant organs (45). However, the trophoblast capacity for proliferation and invasion is limited in extent and duration in order to prevent the excessive destruction of maternal tissues. The pathological mechanisms that cause the disruption of this delicate equilibrium between maternal and fetal tissues largely unknown. The evidence presented here strongly suggests that autocrine production of hemopoietic growth factors may be of importance in the development of choriocarcinomas but further studies are required to determine its precise contribution to this process.

Finally, while the precise role of autocrine secretion of GM-CSF and CSF-1 in the malignant transformation of human trophoblasts remains to be established, our findings and those of others (9,10), certainly warrant the reconsideration of the potential cellular targets of hemopoietic colony-stimulating factors. The normal target range for these cytokines extends well beyond hemopoiesis (a mesenchymal process) to the trophectoderm and normal cells of diverse embryologic origin appear capable of their production. Thus, aberrant expression of either cytokines or receptor may underlie a number of non-hemopoietic malignancies.

Figure 3.1 Expression of CSF-1 receptor mRNA in normal trophoblasts and choriocarcinoma cell lines.

15 μg of total RNA were run on denaturing agarose gels and transfered onto nitrocellulose paper. The filter was hybridized with a 2.8 kb human c-*fms* cDNA probe labled with ^{32}P-dCTP by random oligonucleotide priming as described in methods.

Lane 1: RNA isolated from BeWo cells.
Lane 2: RNA isolated from JEG-3 cells.
Lane 3: RNA isolated from JAR cells.
Lane 4: RNA isolated from normal term trophoblasts

Figure 3.1 Expression of CSF-1 receptor mRNA in normal trophoblasts and choriocarcinoma cell lines.

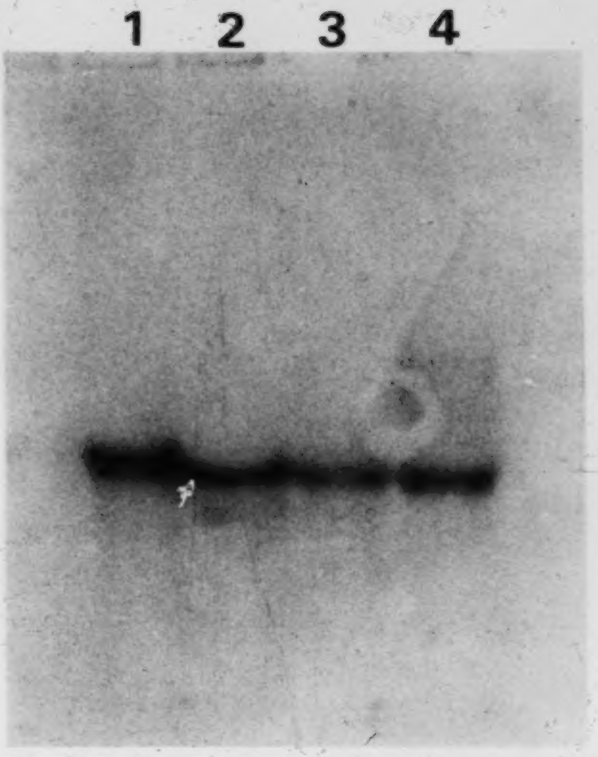

Table 3. 1 Comparison of Unoccupied ^{125}I-CSF-1 Binding Sites on Choriocarcinoma Cell Lines under Saturation Binding Condition at 4° C.

Cell Line	Net High Affinity Binding*	
	cpm/10^5 cells	molecules/cell
BeWo	1,760 ± 43	2,650 ± 65
JEG	1,360 ± 100	2,050 ± 151
JAR	2,130 ± 596	3,210 ± 897

* Determined as detailed in the Methods.

TABLE 3. 2 Effects of Growth Factors on the Cclonogenic Growth of Human Choriocarcinoma Cell Lines.

Cell Line	Source of Growth[1] Factor	Clonogenic Growth[2] (% Control)
BeWo	None	100
	BeWo C.M.	127 ± 2.3
	CSF-1	159 ± 4.8*
	IL-3	108 ± 6.9
JEG-3	None	100
	JEG-3 C.M.	117 ± 6.0
	CSF-1	132 ± 7.0*
	IL-3	134 ± 2.8*
JAR	None	
	JAR C.M.	121 ± 2.9
	CSF-1	154 ± 2.0*
	IL-3	136 ± 2.6*

1. C.M. (media conditioned by the indicated cell line at mid-log phase growth) were used at 10% (v/v). Pure human recombinant CSF-1 was added at 1000 U/mL and recombinant human IL-3 were added at concentrations that maximally stimulated growth of CML cells (see Methods, GM-CSF Assay).

2. Clonogenic assays were carried out in 96 well dishes at 100 cell/well as detailed in the Methods. As typical assay of 6 replicate wells with no added factor, displayed a total of 36 BeWo, 40 JEG-3, and 34 JAR colonies = 100%. Percent values given represent the mean and standard deviation of 9 individual experiments as assessed by the Rantest program (53,54) which also assigned the significant values. *$p < 0.001$

TABLE 3.3 CSF-1 Content in Choriocarcinoma Cell Supernatants and Subcellular Fractions After a 48 hr Incubation.

Cell Type	**CSF-1 Content (Units/compartment)***			
	Supernatant	Cell surface		Intracellular
		receptor bound	trypsin releasable	
BeWo	8,400 ± 1360	1,115 ± 28	1,401 ± 96	162 ±11
JEG-3	2,990 ± 490	515 ± 70	1,158 ± 99	163 ±35
JAR	9,500 ± 500	508 ± 125	1,782 ± 126	161 ±41

* CSF-1 quantitated by RRA as described in the Methods. Values given are the total content in each of the 4 compartments.

TABLE 3.4 Inhibition of the Autonomous Proliferation of BeWo Cells by Anti-CSF-1 Receptor Antibody.*

Antibody[&]	^{3}HTdR Uptake (cpm/well)	Percent Inhibition
none	9,596 ± 943	0
anti-CSF-1R	1,442 ± 177	85
anti-mouse class II	7,446 ± 862	23

[*] Proliferation assessed as ^{3}HTdR uptake during a 4 hr pulse after a 48 hr culture period as detailed in the Methods.

[&] Antibodies were isotype matched (rat IgG2b) and used at a final concentration of 1 ug/mL.

TABLE 3.5 GM-CSF Production by Choriocarcinoma Cell Lines: Detection by CML Growth Specified by Anti-GM-CSF Antibody.

Supernatant	^{3}HTdR Uptake with:*		[GM-CSF]
	NBS Control	Anti-GM-CSF	(U/ml)
rhGM-CSF	67,941 ± 2,713	7,668 ± 1,391	8.0
BeWo-CM (20 x conc.)	33,349 ± 2,416	12,124 ± 664	1.1
JAR	56,271 ± 1,138	32,765 ± 1,416	1.2
JEG	22,094 ± 1,753	14,042 ± 1,850	0.40

* GM-CSF was quantitated as detailed in the Methods. NBS normal bovine serum) was added to control cultures at the same concentration as the bovine anti-human GM-CSF antiserum.

TABLE 3.6 Detection of GM-CSF by CML Growth: Specificity with Anti-GM-CSF Antibody[&].

Added Factor	^{3}HTdR Uptake (cpm/10^4 cells) with	
	NBS Control	Anti-GM-CSF Antibody
Medium	2,345 ± 1	1,426 ± 35
GM-CSF (1 U)	17,844 ± 754	1,977 ± 278
IL-3*	37,554 ± 921	28,164 ± 2,812
CSF-1 (2000 U)	2,675 ± 257	1,523 ± 63

& Carried out as decribed in the legend to Table 5 and the Methods.

* At levels that maximally stimulate CML growth

TABLE 3.7 Inhibition of Choriocarcinoma Cell Line Growth by Anti-GM-CSF Antibody.

Cell Line	^{3}HTdR Uptake (cpm/10^3 cells)		
	Medium (+ NBS)	Anti GM-CSF	Anti GM-CSF + xs GM-CSF
BeWo	4,724 ± 849	1,166 ± 320	5,629 ± 199
JEG	6,526 ± 1,249	1,957 ± 722	6,394 ± 1,021
JAR	26,216 ± 2,356	14,003 ± 3,077	n.d.

BIBLIOGRAPHY

1. Clark S.C. and Kamen R. Science 230:1229-1235, 1897.

2. Stanley E.R., Guilbert L.J., Williams R and Bartelmez S.H. J Cell Biochem 21:151, 1983.

3. Kawasaki E.S *et al* Science 230:291, 1985.

4. Wong G.G. *et al* Cancer cells 3:325, 1985.

5. Van Eewen D.M. *et al* Blood 73:1141, 1989.

6. Cherr C.J. Blood 75:1, 1990.

7. Gearing D.P., King J.A., Gough N.M. and Nicola N. EMBO J 8:3667, 1989.

8. Gorman D.M. *et al* Science 247:324, 1990.

9. Baldwin G.C. *et al*. Blood 73:1033-1037, 1989.

10. Dehdar S. *et al*. PNAS USA 85:9253-9257, 1988.

11. Scheffler J.E. *et al*. J. Cell Biochem. (Suppl) 13C:30, 1989.

12. Rettenmeier C.W, *et al*. J. Clin. Invest. 77:1740-1746, 1986.

13. Bartocci A., Pollard J.W. and Stanley E.R. J. Exp. Med. 164:956-961, 1986.

14. Pollard J.W. *et al.* Nature 330:484-486, 1987.

15. Athanassakis I. *et al.* J. Immunol. 138:37-44, 1987.

16. Fox H. Trophoblastic tumors of the placenta in Pathology of the Placenta (J. Bennington, ed) W.B. Saunders, 1986. pp 369-425.

17. Schniffer M.A., Pomerance W. and Morchels A. Am. J. Obst. Gynec. 90:516-531, 1960.

18. Sporn M.B. and Roberts A.B. Nature 313:745-747, 1985.

19. Schrader J.W. and Crapper R.M. PNAS (USA) 80:6892-6896, 1983.

20. Lang R.A. *et al.* Cell 43:531-542, 1985.

21. Guilbert L.J. and Stanley E.R. J.Immunol. Meth. 42:253, 1981

22. Sherr C.J. *et al.* Cell 41:661-665, 1985.

23. Woolford J., Rothwell V. and Rohrscheneider L. Mol. Cell Biol. 5:3458-3466, 1985.

24. Guilbert L.J., Tynan P.W. and Stanley E.R. J. Cell Biochem. 31:203-216, 1986.

25. Land H., Parada L.F. and Weinberg R.A. Science 222:771-778, 1983.

26. Young D.C. and Griffin J.D. Blood 68:1178-1181, 1986.

27. Scala G.*et al.* J. Immunol. 138:2527-2534, 1987.

28. Rambaldi A. *et al.* J. Clin. Invest. 81:1030-1035, 1988.

29. Kawano M. *et al.* Nature 332:83-85, 1988.

30. Marx J. Science 246:1386-1388, 1989.

31. Browder T.M. *et al.* Mol. Cell. Biol. 9:204-213, 1989.

32. Hapel A.J. *et al.* Cell 25:179-186, 1981.

33. Gazit A. *et al.* Cell 39:89-97, 1984.

34. Laker C. *et al.* PNAS 84:8458-8462, 1987.

35. Wheeler E.F. *et al.* Nature 324:337-380, 1986.

36. Rambaldi A., Young D.C. and Griffin J.D. Blood 1409-1413, 1987.

37. Sieffe C.A. *et al.* Blood 72:1316-1323, 1988.

38. Morgan D.A., Ruscetti F.W. and Gallo R.C. Science 193:1007-1008, 1976.

39. Goustin A.S. *et al.* Cell 41:301-312, 1984.

40. Yagel S. *et al.* Am. J. Obst. Gynec.159:350-361, 1989.

41. Thorens B., Mermod J.J. and Vassali P. Cell 48:671-679, 1987.

42. Horiguchi J., Sariban E. and Kufe D. Mol. Cell. Biol. 8:3951-3954, 1988.

43. Keating M.T. and Williams L.Y. Science 239:914-916, 1988.

44. Garcia Lloret M.I. *et al* (un unpublished data)

45. Beaconsfield P. Science 219:734-739, 1980.

46. Guilbert L.J., Nelson D.J., Hamilton J.A. and Williams M. J. Cell Phy. 115:276-282, 1983.

47. Branch D.R. and Guilbert L.J. J. Tissue Cult. Meth. 10:101-108, 1986.

48. Branch D.R., Turner A.R., and Guilbert L.J. Blood 73:307-311, 1989.

49. Griffin J.D. *et al.* Blood 63:904-911, 1984.

50. Yang Y.-C. *et al.* Cell 47:3-10, 1986.

51. Morrish D.W. *et al* J Clin Endoc Metab 65:1282, 1987.

52. Chomcynski P. and Sacchi N. Anal Biochem 162:156, 1987.

53. Feinberg A.P. and Vogelstein B. Anal Biochem 132:6, 1982.

54. Edgington E.S. in Randomization tests. Second Edition, Marcel Dekker,Inc. New York and Basel (1987).

55. Hooton J.W.L. (Submitted for publication)

CHAPTER 4 GENERAL DISCUSSION & CONCLUSIONS

In this Thesis I investigated the role of the hemopoietic growth factors GM-CSF and CSF-1 as potential regulators of human trophoblast growth and differentiation with the final aim of contributing to the present understanding of some aspects of the molecular biology of pregnancy.

Three major issues raised by the results obtained during the completion of this study need to be addressed: hemopoietic colony-stimulating factors (HCSFs) are trophoblast growth factors; 2) HCSFs are versatile hormones with broader specificity than originally suspected; 3) The response to HCSFs might be a hallmark of embryologically distinct but functionally akin tissues of the body.

1. CSF-1 AND GM-CSF ARE TROPHOBLAST GROWTH FACTORS

The mechanisms that regulate the proliferation and function of the human trophoblast are very poorly understood not only due to the cellular complexity of the materno fetal interphase and to the difficulty of obtaining clearly identified stage specific tissues, but also because of the uniqueness of trophoblast itself, a specialized tissue that exhibits changing patterns of growth, differentiation and function throughout pregnancy (Loke & Whyte, 1983).

There is much circumstantial evidence implicating a role for HCSFs in murine placental development. Pollard *et al.* (1987,1989) have shown that the expression of CSF-1 in the uterus correlates with the appearance of transcripts for its cognate receptor in trophoblastic cells . In related studies, Athanassakis *et al.* (1987) observed that fetally derived murine placental cells proliferated in response to CSF-1 as well as in response to two other macrophage growth factors, GM-CSF and Interleukin-3. While a major flaw in the later studies was the lack of precise identification of the HCSFs responsive cells within the placenta, taken together these findings strongly suggest that HCSFs might be important regulators of trophoblast growth in the mouse.

The evidence linking HCSFs to human placental development was even more vague. It had been reported that whole placenta (Muller *et al.*, 1983) and BeWo, a choriocarcinoma cell line (Rettenmeier et al, 1986), expressed mRNA for c-fms, which encodes the CSF-1 receptor . Recently, it has been shown that placental membranes and malignant trophoblasts expressed high affinity receptors for GM-CSF (Scheffler *et al.*, 1989). No specific effect of either GM-CSF or CSF-1 on trophoblast cells has been reported in the literature, neither their possible influence in the pathogenesis of trophoblastic malignancies has been addressed.

One of the major obstacles encountered in trophoblast research is the lack of adequate methods for their purification and characterization. Only recently, reliable and reproducible techniques have been developed that allow the isolation of large numbers of fairly homogeneous trophoblasts from chorionic villi of term placentas (Kleiman *et al.*, 1986; Morrish & Siy, 1986). Purity is assessed in terms of presence of epithelial (cytokeratin) (Moll *et al.*, 1982) and trophoblastic specific markers (HCG and HPL) and is usually more than 95%, the usual contaminants being macrophages, fibroblasts and endothelial cells (Butterworth & Lokey, 1985). As a consequence of the declining incidence of choriocarcinoma in the western hemisphere and of the lack of major surgery for its treatment, primary tissues for the study of the biology of malignant trophoblasts are not readily available. However, several investigators have developed permanent lines derived from tumors extracted form patients that can, with the reservations applicable to any cell line selected for its enhanced capacity for *in vitro* propagation, be used as models of the transformed state (Patillo & Gey, 1968; Hertz, 1959). Using these two cell populations, i.e. purified villous trophoblasts and choriocarcinoma cell lines, I have addressed some of the many questions concerning the function of HCSFs normal and malignant trophoblast.

As mentioned above, it had been reported in the literature that normal placenta and the BeWo choriocarcinoma cell line expressed CSF-1 receptor mRNA (c-*fms*). Unknown was whether normal trophoblasts also expressed c-*fms* transcripts or whether its expression was a characteristic feature of all malignant trophoblastic cell lines. By Northern Blot analysis, we found that normal trophoblasts and two other choriocarcinoma cell lines (JAR and JEG-3) express a 4 kb c-*fms* mRNA species similar in size and amount to that detected in BeWo and placental tissues (Figure 3.1). In situ hybridization studies confirmed the presence of c-*fms* transcripts on both syncytio and cytotrophoblastic cells of first trimester chorionic villi (Figure 2.1), indicating that a) the detected message in isolated trophoblasts was not due to macrophage contamination of our cell preparation ;b) CSF-1 receptor transcripts are likely to be expressed in trophoblasts throughout pregnancy. These data show that both normal and malignant trophoblasts actively transcribe the CSF-1 receptor gene in a similar manner, and that constitutive expression of c-*fms* mRNA is an inherent property of trophoblastic cells.

We next asked whether trophoblastic cells were capable of translating the c-*fms* message into a functional, CSF-1 binding, cell surface receptor. We found that both normal and malignant trophoblasts specifically bind ^{125}I CSF-1 (Table 2.1 and 3.1). As it is in murine macrophages and human monocytes, binding was irreversible at 4° C and could be competed by pretreatment of the cells with excess unlabeled ligand. There were no significant differences in the total number of receptors per cell expressed by normal trophoblasts (1,600) and choriocarcinoma cells (2,500), indicating that receptor overexpression , an alteration implicated in the pathogenesis of some tumors (Hendler & Ozanne, 1984), is not likely to play a role in trophoblastic transformation.

Do all normal trophoblasts express CSF-1 receptors or is this a characteristic of a particular subset of the whole population? The answer to this question came from autoradiographic studies (Figure 2.2). To allow the recovery of cell surface structures after trypsinization, binding experiments were always performed 18 hours after cells isolation. Microscopic examination of our cell population revealed that during that period a substantial proportion of isolated trophoblasts had migrated towards each other to from multicellular aggregates and some appeared to have fused into syncytial elements, a phenomenon known to occur in serum containing cultures. Overall 66% of the cellular structures were found to express CSF-1 receptors. This percentage increased substantially (88%) if evaluated as a function of the total nuclei present in the culture. While occasional single cells were strongly positive, the highest density of grains was associated with multinucleated elements indicating that expression of CSF-1 receptors increases with differentiation or that CSF-1 receptor bearing cells are more inclined to fuse. Interestingly, young macrophages also express increasing amounts of CSF-1 receptors during their maturation (Guilbert & Stanley, 1986). The physiological function of this quantitative changes in receptor expression is not understood but it has been linked to the ability of the target cells to rapidly uptake and degrade the factor thus regulating its effective concentration in the bloodstream (Bartocci *et al.*, 1987).

Once established that both normal and malignant trophoblasts expressed functional CSF-1 receptors, we next investigated whether this factor could elicit any specific response in trophoblastic cells. Based on the observations of Athanassakis *et al.* (1987) in the murine model and encouraged by the recent reports of the expression of GM-CSF receptors in human placental membranes and choriocarcinoma cells (Scheffler, 1989), we simultaneously studied the response of trophoblastic cells to another macrophage growth factor, GM-CSF. In these experiments we found the first significant difference between normal and malignant trophoblasts. Neither factor had any effect on the proliferation of normal cells whereas CSF-1 enhanced the clonal growth of the three choriocarcinoma cell lines by 1.5 to two fold (Table 3. 2). In normal cells GM-CSF and CSF-1 induced dramatic morphological changes resulting from an increased rate and extension of syncytium formation when compared with control cultures (Figure 2.3). Both CSF-1 and GM-CSF also stimulated the secretion of the placenta specific peptide hormones HCG and HPL (Figure 2.4). No effect on HCG secretion was observed in the malignant cell lines.

Several hypothesis could be forwarded to account for the observed differences in the response to HCSFs between these cell populations.

i) The most obvious explanation is that malignant cells have aberrant patterns of response, even more so when we take into consideration that we are using permanent cell lines that might have a variety of additional genetic alterations. Examples abound in the literature showing that malignant cells respond in a distinct way to their normal counterparts. A most representative example is given by the blasts population of acute myeloid leukemias that proliferate in response to G-CSF and GM-CSF (Pebusque *et al.*, 1988) whereas these factors induce terminal differentiation in normal myeloid cells (Nicola & Metcalf, 1985). Whether this is due to the lack of specific proteins that mediate the differentiation signal including the inability of the malignant cell to synthesize autocrine inhibitory factors or to the abnormal processing of the growth factor-receptor signal remains to be explored.

ii) It may be that the proliferative response observed in choriocarcinomas is the normal response of early placental trophoblasts to these factors and what we are seeing in fact reflects different nature of the stage specific tissues employed. Choriocarcinomas derive from trophoblast populations of the first trimester of pregnancy thus a more adequate comparison should be made with normal cells obtained form early placentas. We did not succeed in isolating pure populations of trophoblasts form first trimester placentas but the question as to whether GM-CSF and CSF-1 are proliferating factors for normal human trophoblasts will have to be readdressed in that context. Alternatively our isolation procedure could have selected for a subset of trophoblastic cells with low proliferative capacity that have initiated their final maturation pathway, and are therefore unable to proliferate under any stimuli.

While the role of GM-CSF and CSF-1 in normal trophoblast proliferation remains to be explored, our experiments clearly demonstrate their importance as trophoblast differentiation factors.

Which is the physiological relevance of the observed HCSFs effects on term trophoblasts?

a) Induction of morphological differentiation: The syncytiotrophoblast (ST) is the main site of synthesis of placental hormones (Kaufman & King, 1982). It follows that agents that induce the differentiation of cytotrophoblasts into ST should cause an increase in the synthetic capacities of the placenta. Indeed, both phenomena occurred concomitantly in the GM-CSF/CSF-1 stimulated cultures. Likewise, epidermal growth factor has been reported to enhance both the *in vitro* syncitialization and the production of hCG and hPL by term trophoblasts (Morrish *et al*, 1987) . Furthermore, morphological differentiation of trophoblastic cells may have profound effects in placental functions other than its protein synthetic properties. The syncytiotrophoblast is the tissue in direct contact with maternal blood through which both nutrients and oxygen are provided to the fetus (Boyd & Hamilton, 1974). Agents that increase the rate and the extent of ST formation may therefore increase the transfer of these vital substances from the mother to the conceptus, independently of their effect on other functions of the placenta

b) Stimulation of hormone synthesis: HCG and HPL are the two major peptide hormones produced by the trophoblast. Early during gestation HCG stimulates the function of the corpus luteum of pregnancy thus allowing the continued secretion of ovarian steroid hormones thought to be necessary for the growth and preparation of the endometrium for implantation (Johanssen & Bosu, 1974); later on, HCG modulates placental steroids. The regulation of HCG secretion is little understood. It has been suggested that the production of

HCG in the early blastocyst is autonomous, independent of maternal stimuli (Mc Cormack & Glasser, 1980). Maternal uterine factors might later have a role since in ectopic pregnancies levels are normal up to day ten after ovulation and then decline to half to one third of the concentration of HCG in normal pregnancies of comparable duration (Landesman & Saxena, 1978). Recently, it has been demonstrated that human uterine tissues express mRNA encoding for CSF-1 (Ringler *et al.*, 1989). In the mouse, injections of HCG stimulates the synthesis of uterine CSF-1 via the ovarian hormones estradiol and progesterone (Bartocci *et al.*, 1986). Therefore, it could be proposed that CSF-1 represents the missing link in a positive feedback mechanism between the uterus, the fetus and the ovary crucial for the normal development of pregnancy. CSF-1 could also indirectly stimulate the growth of first trimester trophoblasts since there is some evidence that HCG might be an autocrine growth stimulator of these cells (Yagel *et al.*, 1989). HPL is thought to be responsible for some of the maternal metabolic changes that occur during pregnancy and perhaps to be a direct stimulator of fetal growth due to its human growth hormone-like (STH) activity (Talamantes *et al.*, 1980). Inasmuch as CSF-1 and especially GM-CSF stimulated the production of HPL by the trophoblast, this could result in enhanced fetal growth.

It could be concluded then that both effects of HCSFs on normal trophoblasts, morphological differentiation and increased peptide hormone production, favor the initiation and maintenance of pregnancy.

In contrast to normal trophoblasts, choriocarcinoma cell lines proliferate autonomously *in vitro*, even at low serum concentrations. One of the mechanisms whereby malignant cells can become independent of exogenous growth factors is by developing the ability of producing their own stimulating substances, a phenomenon generally known as autocrine stimulation (Sporn & Todaro, 1980). Alterations in genes coding for growth factors receptor leading to their aberrant expression in cells synthesizing its agonist activity, to their overexpression in cells that normally express them or to their constitutive activation independently of ligand interaction, can also result in selective proliferative advantage and uncontrolled growth and should be included in any comprehensive definition of autocrine stimulation. Our previous results indicated that normal and malignant trophoblasts expressed similar CSF-1 receptor message (in amount and size) and comparable amount of CSF-1 receptors at the cell surface (Figure 2.1). Previous studies from other laboratories have shown that the CSF-1 receptor present on BeWo cells is biochemically and functionally identical to that of normal monocytes (Rettenmeier *et al.*, 1986). Taken together, these findings suggest that CSF-1 receptor alterations are not likely to play a role in trophoblast transformation. We reasoned that if HGCFs were trophoblast growth factors (whether they stimulated proliferation or differentiation or both) it could be possible that HCSFs mediated self-stimulation of trophoblast proliferation could underlie the uncontrolled growth characteristic of choriocarcinomas. We knew form our data that all cell lines expressed CSF-1 receptors (Table 3.1) and from the literature that at least one of them (JAR) expressed the cognate receptor for GM-CSF. The next step was to determine if the cell lines were able to synthesize CSF-1 and/or GM-CSF. We found that all cell lines produced both HCSFs in culture (Table 3.3 and 3.5). That the autocrine secretion of GM-CSF and CSF-1 was in part responsible for the autonomous growth of choriocarcinomas *in vitro* was further demonstrated by the observation that blocking antibodies to the CSF-1 receptor or neutralizing antibodies to GM-CSF were able to inhibit the growth of the three cell lines by 40 to 60% (Tables 3.4 and 3.5). These result indicate that the association of the secreted factors with their cell surface receptors is important for the transmission of the mitogenic signal. It has been demonstrated that in some autocrine tumors, the association of receptor and its ligand might also occur in intracellular compartments (Browder *et al.*, 1989) a possibility that we cannot exclude since we were unable to totally inhibit the proliferation of the choriocarcinoma cell lines by antibody treatment.

The potential for autocrine stimulation if not unique to tumor cells. Macrophages (Metcalf, 1986; Becker, 1987), T cells (Smith, 1988) and endothelial cells (Quesenberry & Gimbrone, 1980) are able to produce several growth factors to which they are known to respond and there is some evidence that the growth of normal trophoblasts might be regulated by platelet derived growth factor (PDGF) (Goustin *et al.*, 1985) and chorionic gonadotrophin (hCG) in an autocrine manner (Yagel *et al.*, 1989). However, synthesis of self-stimulating factors in normal cells appears to occur mainly after induction by exogenous stimuli and is tightly regulated at the transcriptional and postranscriptional level (Ernst *et al.*, 1989) and tumor cells produce autocrine growth factors constitutively (Young *et al*, 1987). Self stimulated uncontrolled growth could therefore be caused by quantitative differences in growth factor production due to a failure in the normal regulatory mechanisms that restrain their synthesis in normal cell. Can upregulation of HCSFs synthesis explain the autonomous growth of choriocarcinomas? We have detected the presence of low levels of both GM-CSF and CSF-1 in tissue culture supernatants of purified term villous trophoblasts. While we cannot exclude that these HCSFs are in fact secreted by the contaminant fibroblasts or macrophages present in our cell population, it is also possible that the trophoblasts themselves are able to produce CSF-1 and GM-CSF after the proper stimuli. Thus the uncontrolled growth of choriocarcinomas could result from the quantitative differences in HCSFs protein produced rather than from qualitative alterations due to the aberrant expression of the CSF-1 and GM-CSF genes in cells that normally do not secrete the factors but express their respective receptors. It must be emphasized that our results with the malignant cell lines must be reevaluated studying primary tissues since permanent cell lines are likely to have additional genotypic and/or phenotypic characteristics .

In conclusion we have clearly demonstrated that both normal and malignant trophoblasts respond to the macrophage growth factors GM-CSF and CSF-1. Malignant trophoblasts by proliferation and term villous trophoblasts by morphological differentiation and increased hCG and hPL secretion. Unregulated GM-CSF and CSF-1 expression in malignant trophoblasts may play a role in the initiation or maintenance of the transformed phenotype.

Are these the only functions performed by HCSFs during placental development? Most likely not. GM-CSF and CSF-1 have been shown to have pleiotropic effects within the hemopoietic system (Clark & Kamen, 1987). They regulate proliferation, differentiation, and the expression of specialized functions on their target cells as well as they are able to induce the synthesis of several cytokines and other mediators of immune reactions. CSF-1 induces the secretion of plasminogen activators from murine macrophages (Lin & Gordon, 1979), it could also stimulate the synthesis of these proteases in trophoblasts, thus favoring implantation. CSF-1 stimulates monocyte migration. In the placenta, it could enhance the migration of extravillous trophoblasts into maternal tissues allowing their invasion of the uterine arteries , a process thought to be essential for establishing an adequate supply of nutrients and oxygen to the fetus (Fox, 1986). CSF-1 and GM-CSF are known enhancers of the phagocytic and microbicidal capacities of macrophages and granulocytes (Gasson, 1989) therefore, acting on the trophoblast, they could prevent fetal loss due to severe infections. Perhaps even before fertilization , these factors have a role in the physiological changes of the human endometrium that occur in each menstrual cycle. Which is their relationship with LIF (Williams *et al.*, 1988), another hemopoietic cytokine shown to inhibit the differentiation of embryonic stem cells? Do they affect the response to TNF a cytokine shown to inhibit the proliferation of murine trophoblasts *in vitro*? Which is the source of these growth factors within the placenta? The trophoblast? The endometrium? Fetal macrophages or fibroblasts?

In synthesis, many possibilities remain to be explored in this exciting new field of reproductive biology. Hopefully from the understanding of the role of hemopoietic growth factors in normal gestation, new insights will be gained into the mechanisms that condition the various disturbances of pregnancy.

2. HEMOPOIETIC COLONY STIMULATING FACTORS ARE VERSATILE CYTOKINES

While other hemopoietic regulators, such and interleukin-1, tumor necrosis factor and interleukin-6 are known to have effects in a wide variety of cells (Mizel, 1989), the response to the colony-stimulating factors has been traditionally considered to be restricted to hemopoietic cells of the myeloid lineage (Metcalf, 1986). Recently it has been reported that cell lines of non hemopoietic origin express receptors for GM-CSF and some are able to proliferate in response to this factor (Baldwin *et al.*, 1989; Dehdar *et al.*, 1988). Small cell lung carcinoma cell lines express a 84 kd high affinity receptor for GM-CSF identical to that present on normal phagocytes. Two osteosarcomas and one breast carcinoma cell line were shown to proliferate in response to GM-CSF when cultured under serum free conditions . These factors also stimulated the growth of a SV-40 transformed fibroblast-like cell line derived from bone marrow stroma (Dehdar *et al*, 1988) . Our own results in the choriocarcinoma cell lines show that the growth of malignant cells of epithelial origin is autocrinally stimulated by both GM-CSF and CSF-1. Taken together these findings clearly indicate that HCSFs may affect the proliferation of transformed cells of diverse embryologic origin and may be implicated in the pathogenesis of a variety of non hemopoietic malignancies either by paracrine stimulation from factors secreted by macrophages or endothelial cells in close association with neoplastic cells or by self-stimulation of growth by tumors both secreting and responding to HCSFs. These observations are not only important for the understanding of the pathogenesis of some human malignancies but also warrant further studies as to whether HCSFs can be safely used as therapeutic agents in the treatment of radiation or drug induced myelosuppression in non-hemopoietic malignancies (Brandt *et al.*, 1988).

HCSFs are also likely to play a role as physiologic regulators outside the hemopoietic system. Our results clearly indicate that HCSFs have profound effects on trophoblast differentiation. Lately, it has been shown that normal umbilical vein endothelial cells proliferate and migrate in response to GM-CSF and to G-CSF. These initial findings suggest that HCSFs have a broader specificity than originally suspected and open new areas of research for these versatile cytokines as mediators of non-hemopoietic functions.

3 THE RESPONSE TO HCSFs IS DETERMINED BY FUNCTIONAL REQUIREMENTS

The evidence at present is that trophoblasts and endothelial cells, and possibly fibroblasts (Zucalu *et al.*, 1986; Dehdar *et al.*, 1988), appear to respond to similar patterns of HCSFs as mononuclear phagocytes. Is there any link between these embryologically distinct cell types? Macrophages and trophoblasts share a number of biological properties. They both are strongly esterase positive and express Fc receptors (Wild, 1983). Trophoblasts have recently been shown to synthesize interleukin-1 (Maine *et al*, 1987), a characteristic monokine. Macrophages secrete proteases (Unkeless *et al.*, 1974) and migrate through tissues (Wang *et al.*, 1988), as trophoblasts do (Yagel*et al*, 1988) and both cell types are capable of phagocytosis. Endothelial cells resemble macrophages in many aspects as illustrated by the previously widespread use of the expression "reticuloendothelial system" to identify both cell populations. Mononuclear phagocytes, endothelial cells and fibroblasts are major producers of inflammatory cytokines that mediate immune reactions and the three cell types coordinately interact in the processes of wound healing and tissue remodeling. HCSFs mediated common regulatory mechanisms could therefore reflect related functional requirements, independently of embryologic origin.

Aitken R.J., Beaconsfield R. and Ginsburg J. Origin and formation of the placenta. In: Placenta - A neglected experimental model. (P. Beaconsfield and P. Villee, eds.) Pergamon Press, 1979. pp 152-163.

Asch R.H., Fernandez C.O. and Pauerstein C.J. Immunodetection of human chorionic gonadotrophin-like substance in human sperm. Fertil. Steril. 28:1258-1260, 1977.

Athanassakis I., Bleackley C.R., Paetkau V., Guilbert L.J., Barr P.J. and Wegmann T.G. The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells J. Immunol. 138:37-44, 1987.

Azoulay M., Webb C.G. and Sachs L. Control of hematopoietic cell growth regulators during mouse fetal development. Mol. Cell. Biol. 7:3361-3363, 1987.

Bagshawe K.D. Risk and prognostic factors in trophoblastic neoplasia. Cancer 38:1373-1385, 1976a.

Bagshawe K.D. Endocrinology and the treatment of trophoblastic tumors. J. Clin. Pathol 29:140-149, 1976b.

Baldwin C.C.G., Gasson J.C., Kaufman S., Quan S., Williams R.E., Avalos B., Gazdar A.F., Golde D.W. and DiPersio J.F. Non-hematopoietic tumor cells express functional GM-CSF receptors. Blood 73:1033-1037, 1989.

Bartelmez S.H. and Stanley E.R. Synergism between hemopoietic growth factors detected by their effects on cells bearing receptors for a lineage-specific hemopoietic growth factor. Assay for hemopoietin 1. J. Cell. Physiol. 122:370-375, 1985.

Bartocci A., Mastrogiannis D.S., Migliorati G., Stockert R.J., Wolkoff A.W. and Stanley E.R. Regulation of colony-stimulating factor-1 during pregnancy. J. Exp. Med. 164:956-961, 1986.

Beaconsfield P., Birdwood G. and Beaconsfield R. The Placenta. Sci. American 243:95-102, 1980.

Billiau A., Van Damme J., Ceuppens J. and Baroja M. Interleukin-6, a ubiquitous cytokine with paracrine as well as endocrine functions. In: Lymphokine Receptor interactions (D. Fradelizi and J. Bertoglio, eds.) London: John Libby Eurotext Ltd, 1987. pp 133-142.

Bishop J.M. The molecular genetics of cancer. Science 235:305-311, 1987.

Bohem K.D., Kelley M.F., Ilan J. and Ilan J. The interleukin-2 gene is expressed in the syncytiotrophoblast of the human placenta. Proc. Natl. Acad. Sci. (USA) 86:656-660, 1989.

Bohn H. Detection and characterization of pregnancy specific proteins in the human placenta and their quantitative immunochemical determination in sera from pregnant women. Arch. Gynek. 210:440, 1971.

Boyd J.D. and Hamilton W.J. The human placenta. Mac Millan, 1974.

Branch D.R. and Guilbert L.J. Synergistic stimulation of macrophage proliferation by the monokines tumor necrosis factor alpha (TNF) and colony-stimulating factor-1 (CSF-1). Blood 73:307-311, 1989.

Broudy V.C., Kaushansky K., Segal J.M., Harlan J.M. and Adamson J.W. Tumor necrosis factor alpha stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. (USA) 83:7467-7470, 1986.

Bulmer J.N. and Sutherland C.A. Bone marrow origin of endometrial granulocytes in the early human placental bed. J. Rep. Immunol. 5:383-387, 1983.

Bulmer J.N. and Johnson P.M. Macrophage populations of the human placenta and amniochorion. Clin. Exp. Immunol. 57:393-403, 1984.

Burgess A.W., Wilson E.M.A. and Metcalf D. Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood 49:573-583, 1977.

Cannistra S.A. and Griffin J.D. Regulation of the production and function of granulocytes and macrophages. Semin. Hematol. 25:173-181, 1988.

Carpenter G. Polypeptide growth factors receptors. Ann Rev Biochem 56:881-902, 1987.

Chard T. Normality and abnormality. In: Placental hormone assays in the evaluation of fetal well-being. (A. Klopper, ed.) Edinburgh: Churchill Livingston , 1976.

Chou J.C. Establishement of clonal human placental cells synthesizing human chorionic gonadotrophin. Proc. Natl. Acad. Sci. (USA) 75:1854-1858, 1978.

Chou J.C., Robinson J.C. and Wang S.S. Effects of sodium butyrate on synthesis of human chorionic gonadotrophin in trophoblastic and non-trophoblastic tumors. Nature 268:543-544, 1977.

Clark S.C. and Kamen R. The human hemopoietic colony-stimulating factors. Science 236:1229-1237, 1987.

Cukrova V. and Hrkal Z. Purification and characterization of granulocyte-macrophage colony-stimulating factor from human placenta. J. Chromatogr. 413:242-246, 1987.

Culouscou J.M., Remacle-Bonnet M.M. Pommier G., Rance R.J. and Depieds R.C. Immunosuppresive porperties of the human placenta: study from supernatants from short term syncytiotrophoblast cultures. J. Reprod. Immunol. 9:33, 1986.

Demetri G.D., Zenzie B.W., Rh[illegible]nwald J.G. and Griggin J.D. Expression of colony-stimulating factor genes by normal and malignant mesothelioma cell lines *in vitro*. Blood 74:940-946, 1989.

Dickman Z., Dey S.K. and Gupta J.S. A new concept: control of early pregnancy by hormones originating in the preimplantation embryo. Vitam. Horm. 34:215-242, 1976.

Dickman W.J. and Cauchi M.N. Lymphocyte-induced stimulation of human chorionic gonadotrophin production by trophoblastic cells *in vitro*. Nature 271:377-378, 1978.

Dipersio J.F., Billing P., Kauffman S., Eghtesady P., Williams R.E. and Gasson J.C. Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. J. Biol. Chem. 263:1834, 1988.

Eaves A.C. and Eaves C.J. Erythropoiesis in culture. In: Clinics in Hematology (E.A. Mc Culloch, ed.) Toronto: W.B. Sanders, 1985. pp 371.

Enders A.C. Fine structure of anchoring villi of the human placenta. Am. J. Anat. 122:419-451, 1968.

Ernst T.J., Ritchie A.R., Demetri G.D. and Griffin J.D. Regulation of granulocyte and monocyte colony-stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post-transcriptional level. J. Biol Chem. 264:2700, 1989.

Fant M., Munro H. and Moses A.C. An autocrine/paracrine role for insulin growth factors in the regulation of human placental growth. J. Clin. Endoc. Metab. 63:499-505, 1986.

Feinman M.A., Kliman H.J. and Strauss J.F. 8-bromo 3',5' adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. J. Clin. Endoc. Metab. 63:1211-1217, 1986.

Fisher S.J., Leitch M.S., Kantor M.S. and Kramer R.H. Degradation of extracellular matrix by the trophoblastic cells of first trimester human placentas. J. Cell. Biochem. 27:31-41, 1985.

Fox H. The placenta in abortion. In: Pathology of the placenta (H.H. Fox and J.L. Bennington, eds.) W.B. Saunders, 1978. pp 258-269.

Fox H. Pathology of the placenta. Clinics in Obstetrics and Gynecology 13:501-520, 1986.

Friesen H. Lactation induction by human placental lactogen and cortisone acetate in rabbits. Endocrinology 79:212-216, 1966.

Gardner R.L. and Rossant J. Determinatioon during embryogenesis. In: Embryogenesis in mammals. Ciba Found. Symp. 40. Elseevier: North Holland Publishers, 1976. pp 5-18.

Gasson J.C., Weisbart R.H., Kaufman S.E., Clark S.C., Hewick R.M., Wong G.G. and Golde D.W. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. Science 226:1339. 1984.

Gerbie A.B., Hathaway H.H. and Brewer J.I. Autoradiographic analysis of normal trophoblast proliferation. Am. J. Obst. Gynec. 100:640-650, 1968.

Glasser S.R. and Mc Cromack S.A. Cellular and molecular aspects of decidualization and implantation. In: Proteins and Steroids in early mammalian development. (H. Beier and P. Karlson, eds.) Springer Verlag, 1980.

Golde D.W., Quan S.G. and Cline M.G. Human T lymphocyte cell line producing colony-stimulating activity. Blood 52:1068, 1978.

Goldstein D.P. and Berkowitz R.S. Gestational trophobalstic neoplasms. Clinical principles of diagnosis and management. Philadelphia: W.B. Saunders, 1982. pp 188-288.

Gore H. and Hertig A.T. Problems in the histologic interpretation of the trophoblast. Clinics in Obstetrics and Gynecology 10: 269, 1970.

Goustin S.A., Bertzholtz C., Pfeiffer-Ohlsson S., Persson H., Rydnert J., Bywater M., Holmren G., Heldin C., Westermark B. and Ohlsson R. Coexpression of the sis and myc proto-oncogenes in developing human placenta suggests autocrine control of trophoblast proliferation. Cell 41:301-312, 1985.

Grabstein K.H., Urdal D.L., Tushinski R.J., Mochizuki D.Y., Price V.L., Cantrell M.A., Gillis S. and Conlon P.J. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science 232:506-508, 1986.

Gruenwald P. The placenta and its maternal supply line (P. Gruenwald, ed.) Baltimore: University Park Press, 1976. pp 1-12.

Grumbach M.M., Kaplan S.L. and Vinik A. Human chorionic somatotrophin. In: Methods in investigative and diagnostic endocrinology. (S.A. Berson and R.A. Yalus, eds.) Amsterdam: North Holland Publishers, 1973.

Halban J. Die imeresekretion von ovarium und placenta und ihre bedeutung fur die function der milchendere. Arch. Gynek. 75:353, 1905.

Handwerger S., Conn P.M., Barrett J. and Golander A. Human placental lactogen release *in vitro*: paradoxical effects of calcium. Am. J. Physiol. 240:E550, 1981.

Heikinheimo M., Gahmberg C.G., Bohn H. and Anderson L.C. Oncoplacental protein SP1-A constitutive and inducible late differentiation marker of the human myelomonocytic lineage. Blood 70:1279-1283, 1987.

Hertz R. Choriocarcinoma of women maintained in serial passage in hamster and rat. Proc. Soc. Exp Biol. Med. 102:77-80, 1959.

Hertz R. Spectrum of gestational trophoblastic disease. In: Choriocarcinoma and related gestational trophoblastic tumors in woman. New York: Raven Press. 1978, pp 13-22.

Hill J.A., Himovici F and Anderson D.J. Products of activated lymphocytes and macrophages inhibit mouse embryo development *in vitro*. J. Immunol. 139:2258-2254, 1987.

Horiguchi J., Warren M.K. and Kufe D. Expression of macrophage colony-stimulating factor-1 (CSF-1) in human monocytes treated with granulocyte-macrophage colony-stimulating factor. Blood 69:1259-1261, 1987.

Hoshina M., Nishio A., Bo M., Boime I. and Mochizuki M. The expression of the oncogene fms in human chorionic tissue. Acta Obstet. Gynec. (Japan) 37:2791-2798, 1985.

Hung W., Blizzard R.M., Migeon C.J., Camacho A.M. and Nyhan W.L. Precocious puberty in a boy with hepatoma and circulatory gonadotrophin. J. Pediatr. 63:895, 1963.

Inskeep E.K., Wilson L., Butler W.R., Dierschke D.J., Fritz G.R. and Knobil E. Endometrila secretion of prostaglandin during the ovarian cycle and early pregnancy. In: The Endometrium (F.A. Kimball, ed.) SSP Medical and Scientific Books, 1983, pp 287-310.

Javadpour J. Current concepts in management of testicular tumor. Urol. Survey 28:89-96, 1978.

Johanssen E.D., Bosu W.T.K. The function of the corpus luteum in primates. In: Physiology and genetics of reproduction (E.M. Cpountinho and F. Fuchs, eds.) New York: Plenum Press, 1974. pp 343-352.

Joint Projects for study of choriocarcinoma and hydatidiform mole in Asia. Geographic variation in the occurrence of hydatidiform mole and choriocarcinoma. Ann. N.Y. Acad. Sci. 80:178-195, 1969.

Josimovich J.B. Maintenance of pregnancy in the rat by synergism between human placental lactogen and hCG. Endocrinology 83:707, 1966.

Josimovich J.B. Placental lactogen and pituitary prolactin. In: Endocrinology of pregnancy. (A. Klopper and F. Fuchs, eds.) Philadelphia: Harper and Row, 1981. pp 144-160.

Josimovich J.B. and Mac Laren J.A. Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. Endocrinology 71:209-214, 1962.

Jovanovic I., Landesman R. and Saxena B.B. Screening for twin pregnancy. Science 198:738-749, 1977.

Jovanovic I., Dawood M.Y. and Saxena B.B. Hormonal profile as a prognostic index of early threatened abortion. Am. J. Obstet. Gynecol. 130:274-278, 1978.

Kaufmann P. and King B.F. Structural and functional organization of the placenta. Basel: S. Karger. 1982.

Kearns M. and Lala P.K. Bone marrow origin of decidual cell precursor in the pseudopregnant rat uterus. J. Exp. Med. 155-1537, 1982.

Kliman H., Feinman M.A. and Strauss J.F.III Differentiation of human cytotrophoblasts in syncytiotrophoblasts in culture. Troph. Research 2:407-421, 1987.

Kurkland J.I., Piles L.M., Ralph P., Bockmann R.S. and Moore M.A.S. Induction of prostaglandin E in normal and neoplastic macrophages. Role of colony-stimulating factor(s) distinct from effects on myeloid cell proliferation. Proc. Natl. Acad. Sci. (USA) 76:2326-2330, 1979.

Land H., Parada L.F. and Weinberg R.A. Cellular oncogenes and multistep carcinogenesis. Science 222:771-777, 1983.

Landesman R. and Saxena B.B. Results of the first 1000 radioreceptor assays for the determination of human chorionic gonadotrophin... A new, rapid, reliable and sensitive pregnancy test. Fertil. Steril. 27:357-362, 1976.

Landesman R. and Saxena B.B. Diagnosis of ectopic pregnancy by radioreceptor assay for chorionic gonadotrophin. Lancet 8067:772-774, 1978.

Lin H.S. and Gordon S. Secretion of plasminogen activator by bone marrow dereived mononuclear phagocytes and ites enhancement by colony-stimulating factor. J. Exp. Med. 150:231-245, 1979.

Macaron C., Famuyiwa O. and Singh S.P. *In vitro* effect of dopamine and pimozide on human chorionic somatomammotrophin (hCS) secretion. J. Clin. Endoc. Metab. 47:168, 1978.

Main E.K., Strizki J. and Schochet P. Placental production of immunoregulatory factors: Trophoblast is a source of interleukin-1. Trophoblast Research 2:149-160, 1987.

Mano T. and Chou J.C. Regulation of human chorionic gonadotrophin synthesis in cultured human placental cells by glucocorticoids. Endocrinology 109:888-892, 1981.

Maoz H., Kaiser N., Halimi M., Barak V., Haimovits A., Weinstein D., Simon A., Yagel S., Biran S. and Treves A. The effect of estradiol on human myelomonocytic cells I. Enhancement of colony formation. J. Reprod. Immunol. 7:325-335, 1985.

Martin L. and Finn C.A. Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus. J. Endoc. 41:363-371, 1968.

Mc Cormack S. and Glasser S.R. Hormone production by rat blastocysts and mid-gestation trophoblast *in vitro*. In: The Endometrium (F.A. Kimball, ed.) SPP Medical and Scientific Books, 1983. pp 145-190.

Mercanti D., Luzzatto E., Ciotti M.T. and Levi G. Mitogenic effect of a human placental factor on astrocytes and glial precursors. Exp. Cell Res. 168:182-190, 1987.

Metcalf D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood 67:257-263, 1986.

Mizel S.B. The interleukins. FASEB J. 3:2379-2388, 1989.

Morrish D.W., Bhardwaj D., Dabbagh L.K., Marusyk H. and Siy O. Epdermal growth factor induces the differentiation and secretion of human chorionic gonadotrophin in normal human placenta. J. Clin. Endoc. Metab. 65:1282-1290, 1987.

Moscatelli D.A., Presta M., Mignatti P., Mullins D.E., Crowe R.M. and Rifkin D.B. Purification and biological activities of an angiogenesis factor from human placenta. Anticancer Research. 6:861-863, 1986.

Moscatelli D., Presta M. and Rifkin D.B. Purification of a factor from human placenta that stimulates capillary endothelial cells protease production, DNA synthesis and migration. Proc. Natl. Acad. Sci. (USA) 83:2091-2095, 1986.

Muller R., Slamon D.J., Adamson E.D., Tremblay J.M., Muller D., Cline M.J. and Verma I.M. Transcription of c-*onc* genes c-*ras* and c-*fms* during mouse developement. Mol. Cell. Biol. 3:1062-1069, 1983.

Namen A.E., Lupton S., Hjerrild K., Wignall J., Schmierer A., Mosley B., March C.J., Urdal D., Gillis S., Cosman D. and Goodwin R.G. Stimulation of B cell progenitors by cloned murine interleukin-7. Nature 333:571-573, 1988.

Nehemiah J.E., Schnitzer J.A, Schulman H. and Novikoff A.B. Human chorionic trophoblasts, decidual cells and macrophages: histochemical and electron microscopic study. Am. J. Obstet. Gynecol. 140:261-26, 1981.

Nielsen P.V., Pedersen H. and Kampmann E.M. Absence of human placental lactogen in an otherwise uneventful pregnancy. Am. J. Obstet. Gynec. 135:322-324, 1979.

Ohama K., Kajii T., Okamoto E., Fujuda Y., Imaizmi K., Tsukahara M., Kobayashi K. and Hagiwara K. Dispermic origin of XY moles. Nature 292:551-552, 1981.

Panigel M. Anatomy and morphology of the placenta. Clinics in Obstetrics and Gynecology 13:421-445, 1986.

Park W.W. Choriocarcinoma: A study of its pathology. London. Heinemann, 1971.

Pasqualini J.R. and Kincl F.A. Hormones and the fetus. Volume 1. Pergamon Press, 1977.

Patillo R.A. and Gey G.O. The establishment of a cell line of human hormone synthesizing trophoblstic cells *in vitro*. Cancer Res. 28:1231-1236, 1968.

Paul W.E. and Ohara J. B cell stimulatory factor/interleukin-4. Annu. Rev. Immunol. 5:429-460, 1987.

Pijneborg R., Bland J.M., Robertson W.B., Dixon G. and Brosens I. The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy. Placenta 2:303-316, 1981.

Pollard J.W., Arceci R.J., Bartocci A., and Stanley E.R. Colony-stimulating factor-1: a growth factor for trophoblasts? In: Proceeding of Third Banff Conference in Reproductive Immunology, 1989.

Pollard J.W., Bartocci A., Arceci R., Orlofsky A., Ladner M.B. and Stanley E.R. Apparent role for the macrophage growth factor, CSF-1, in placental developement. Nature 330:484-486, 1987.

Psychoyos A. Hormonal control of implantation. Vit. Horm. 31:201-256, 1974.

Queenan J.T., Lao L.Ch., Arboleda C.E., Ulloa Aguirre A., Golos T.G., Cines D.B. and Strauss J.F.III Regulation of urokinase type plasminogen activator production in cultured human cytotrophoblasts. J. Biol. Chem. 262:10903-10906, 1987.

Ramsey E.M. The placenta of laboratory animals and man (J.D. Ebert, ed.) Holt Rinehart and Winston, 1975.

Redman C.W.G. Immunology of the placenta. Clinics in Obstetrics and Gynecology 13:469-499, 1986.

Rennick D., Young G., Gemmell L. and Lee F. Lypopolysaccharide and interleukin-1 inducible production of colony-stimulating factors. Blood 69:682-686, 1987.

Reisbach G., Sinderman J., Kremer J., Hultner L. and Dormer P. Macrophage Colony Stimulating Factor expression in spontaneously outgrown EBV-B cell lines and activated normal B lymphocytes. Blood 74:959-964, 1989.

Rettenmeier C., Sacca R., Furman W.L., Roussel M.F., Holt J.T., Nienhuis A.W., Stanley E.R. and Sherr C.J. Expression of the human c-*fms* oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. J. Clin. Inves. 77:1740-1746, 1986.

Robertson W.B. Uteroplacental vasculature. J. Clin. Pathol. (Supp) 29:10, 1976.

Robertson W.B., Brosensn I. and Dixon G. The ultrastructure of the human placental bed. J. Pathol. 112:203-214, 1974.

Rosal L., Saxena B.B. and Landesman R. Application of the radioreceptor assay for hCG in the diagnosis of early abortion. Fertil. Steril. 26:1105,1975.

Roussel M.F., Downing J., Rettenmeier C.W. and Sherr C.J. A point mutation in the extracellular domain of the human CSF-1 receptor (c-*fms* proto-oncogene) activates its transforming potential. Cell 55:979-988, 1988.

Saxena B.B. Human chorionic gonadotrophin. In: Endocrinology of pregnancy (F. Fuchs and A. Klopper, eds.) Philadelphia: Harper and Row, 1983. pp 50-71.

Saxena B.B. and Landesman R. Diagnosis and management of pregnancy by the radioreceptor assay of hCG. Am. J. Obstet. Gynec. 131:97, 1978.

Scheffler J.T., Fleissner L., Seeling G., Tattanahalli L. and Trotta P. Characterization of the human granulocyte-macrophage receptor from human placenta. J. Cell. Biochem. (Supp) 13C:30, 1989.

Shen S.J., Wang C.Y., Nelson K.K., Jansen M. and Ilan J. Expression of insulin-like growth factor II in human placentas from normal and diabetic pregnancies. Proc. Natl. Acad. Sci. (USA) 83:9179-9182, 1986.

Sherman M. Implantation of mouse blastocysts *in vitro*. In: Methods in mammmalian reproduction. (J.C. Daniel, ed.) New York, Academic Press, 1978.

Sherr C.J., Rettenmeier C.W., Sacca R., Roussel M.F., Look A.T. and Stanley E.R. The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor CSF-1. Cell 41:665-676, 1985.

Silberstein D.S., Owen D.F., Gasson J.C., Di Persio J.F., Golde D.W., Bina J.C., Soberman R., Austen K.F. and David J.R. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by recombinant granulocyte-macrophage colony-stimulating factor. J. Immunol. 137:3290, 1986.

Smith K. Interleukin-2: inception, impact and implications. Science 240: 1169-1176, 1988.

Sporn M.B. and Todaro G.J. Autocrine secretion and malignant transformation of cells. N. Eng. J. Med. 303:771-777, 1980.

Stanley E.R., Guilbert L.J., Tushinski R.J. and Bartelmez S.H. CSF-1, a mononuclear phagocyte lineage-specific hemopoietic growth factor. J. Cell. Biochem. 21:151-159, 1983.

Stevens V.C. Immunization with hormones. In: Reproduction research. Amsterdam: North Holland Publishers, 1975. p 217.

Strickland S. and Reich E. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-240, 1976.

Thorens B., Mermod J.J. and Vassalli P. Phagocytosis and inflamatory stimuli induce GM-CSF mRNA in macrophages through post-transcriptional regulation. Cell 48:671-679, 1987.

Tsai S., Emerson S.G., Sieff C.A. and Nathan D.G. Isolation of a human stromal cell strain secreting hemopoietic growth factors. J. Cell. Physiol. 127:137, 1979.

Vaitukaitis J.L. Changing placental concentrations of hCG and its subunits during gestation. J. Clin. Endoc. Metab. 38:755-760, 1974.

Vaitukaitis J.L. and Ebersole E.R. Evidence for altered synthesis of human CG in gestational trophoblastic tumors. J. Clin. Endoc. Metab. 42:1048-1052, 1976.

Vaitukaitis J.L. Glycoprotein hormones and their subunits. Immunological and biological characterization. In: Structure and function of gonadotrophins (K.W. McKerns, ed.) New York: Plenum Press, 1978. p 339.

Varma S.K.L., Dawood M.Y., Haour F., Channing C.P. and Saxena B.B. Gonadotrophin-like substance in the preimplanted rabbit blastocyst. Fertil. Steril. 31:68, 1979.

Wake N., Seki T., Fujita H., Okubo F., Sakai K., Okuyama K., Hayashi H., Shiina Y., Sato H., Kuroda M. and Ichinoe K. Malignant potential of homozygous and heterozygous complete moles. Cancer Research 44:1226-1330, 1984.

Wake N., Takagi N. and Sasaki M. Androgenesis a cause of hydatidiform mole. J. Natl. Cancer Inst. 60:51-53, 1978.

Warren M.K. and Ralph P. Macrophage colony-stimulating factor stimulates human monocyte production of interferon, tumor necrosis factor and colony-stimulating activity. J. Immunol. 137:2281-2285, 1988.

Wegmann T.G. Fetal protection against abortion: is it immunosuppresion or immunostimulation? Ann. Immunol (Inst. Pasteur) 136D:309-312, 1984.

Weisbart R.H., Golde D.W., Clark S.C., Wong G.G. and Gasson J.C. Human granulocyte-macrophage colony-stimulting factor is a neutrophil activator. Nature 314:361363, 1985.

Weiss T.J., Janson P.O., Porter K.J. and Seamark R.F. Effects of human chorionic gonadotrophin on the release of cyclic AMP and progesterone from the sheep ovary at different stages of the estrous cycle. Acta Endoc. 89:159-165, 1978.

Wing J.N., Ampel A., Waheed A. and Shadduck R.K. Macrophage colony-stimulating factor enhances the capacity of murine macrophages to secrete oxygen reduction products. J. Immunol. 135:2052-2055, 1985.

Wong G.G., Temple P.A., Leary A.C., Witek-Gianotti J.S., Yang Y.C., Ciarletta A.B., Chung M., Kriz R., Kaufman R.J., Ferenz C.R., Sibley B.S., Truner K.J., Hewick R.M., Clark S.C., Yanie N., Yokota H., Yamad M., Saito M., Motoyoshi K. and Takaku F. Human CSF-1: molecular cloning and expression of a 4 kb cDNA encoding the human urinary protein. Science 235:1504-1508, 1987.

Woolford J., Rothwell V. and Rohrschneider L. Characterization of the human *c-fms* gene product and its expression in cells of the monocyte-macrophage lineage. Mol. Cell Biol. 5:3458-3466, 1985.

Wu M., Cini J. and Yunis A. Purification of a colony-stimulating factor from cultured pancreatic carcinoma cells. J. Biol. Chem. 254:6226-6231, 1979.

Wynn R.M. Immunological implications of comparative placental ultrastructure. In: The Biology of the blastocyst (R.J. Blandau, ed.) Chicago: University of Chicago Press, 1971.

Yagel S., Parhar R.S., Jeffrey J.J. and Lala P.K. Normal nonmetastatic human trophoblast cells share *in vitro* invasive properties of malignant cells. J. Cell. Physiol. 136:455-462, 1988.

Yoshimoto Y., Wolfsen A.R., Hirose F. and Odell W.D. Human chorionic gonadotrophin-like material: presence in normal human tissue. Am. J. Obstet. Gynec. 134:729-734, 1979.

Young D.C., Wagner K. and Griffin J.D. Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. J. Clin. Invest. 79:100-104, 1987.

Zeilmaker G.H. and Verhamme CM. Luteotropic activity of ectopically derived rat blastocysts. Acta Endocrinologica 88:589, 1978.